Metabolic Interdependence of Obligate Intracellular Bacteria and Their Insect Hosts†

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INTRODUCTION

Intracellular Bacteria of Eukaryotes: Parasites and Mutualists

The establishment of intimate interactions between two living cells culminating in the stable integration of one cell into another has resulted in major milestones during evolution of life on Earth, as proposed already several decades ago in the sequential endosymbiont theory. Based on earlier suggestions made by scientists including Mereshkovsky, in this theory Lynn Margulis suggested a prokaryotic origin of cell organelles such as mitochondria and chloroplasts (69, 73). In fact, facultative or obligate intracellular bacteria can be found throughout the tree of life from protists to plants and animals (19). Moreover,

the first stable intracellular symbiotic association of one prokaryote within another was recently described (132) and adds to several examples of parasitic bacterium-bacterium interactions exemplified by the ectoparasite *Bdellovibrio* (97) and the facultative intracellular pathogen *Daptobacter*, which is thriving within the cytosol of other bacteria (38, 70). Within an animal host cell, the bacteria can reside in two different compartments. Either they can be localized to a vacuole which may be derived from a phagosome formed during engulfment of the bacteria, or they may colonize the host cell cytosol (36, 88). Because of their medical importance and the possibility of cultivating most of them in vitro, the best characterized bacteria with the ability to survive and multiply within eukaryotic cells are facultative intracellular pathogens of mammals and humans. Examples of bacteria able to multiply inside a vacuole include *Salmonella enterica* serovar typhimurium, *Legionella pneumophila*, *Coxiella burnetii*, *Francisella tularensis*, *Mycobacterium tuberculosis*, and obligate intracellular *Chlamydia* spp., whereas *Listeria monocytogenes*, *Shigella flexneri*, enteroinvasive *E. coli* and some *Rickettsia* species are able to enter and

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replicate in the cytosol of mammalian cells. Although these bacteria need specific (virulence) factors to recognize, invade, and multiply within the eukaryotic cells, for most of them the intracellular phase is transient. This intracellular location may be aimed mainly at the exploitation of host metabolites in order to support bacterial multiplication in a relatively safe host compartment devoid of several potent host defense mechanisms. Moreover, the intracellular state may contribute to the dissemination of the bacteria within the host and, after evading the host cells, their release into the environment or direct transmission to another host organism (28, 37).

However, for the maternally transmitted mutualistic bacteria of insects described in this review, the intracellular phase is an absolute requirement, and no or only short extracellular phases during the development of their host animals may occur during their life cycle. In contrast to pathogens, these bacteria reside in specialized cells, the bacteriocytes, which are provided by the animal hosts and apparently are part of the developmental program of these animals during embryogenesis and larval development (13). The stable integration of these bacteria into a eukaryotic host has required a major adaptation of the bacterial metabolism to that of the host cell. As a consequence, these bacteria so far cannot be cultivated in vitro, probably due to their long-lasting adaptation to their intracellular life-style for the last 250 million years or so (79). Similar to several obligate pathogens, many of these obligate intracellular endosymbionts have extraordinary genome features including an extremely reduced genome size of only 450 to 800 kbp and a correspondingly small coding capacity (34, 133). Accordingly, it is likely that the intimate relationship of these bacteria with their host cells may have enabled or enforced a very significant reduction in the metabolic potential of the bacteria, because redundant metabolic pathways could have been sorted out without damage or, alternatively, deleterious combinations of metabolic reactions of the two organisms or pathways leading to production of toxic metabolites have had to be eliminated or redirected.

In the present review we describe some basic principles of such adaptive events by using the examples of several mutualistic bacteria residing in bacteriocytes of insects. Such bacteriocyte symbioses are quite frequent in several insect orders including Homoptera, Hymenoptera, and Coleoptera (14). The symbiotic interaction is obligate for both partners, since the bacteria cannot be cultivated in vitro and curing the host of their companions has severe consequences for survival and/or reproduction of the animals (23, 25, 138). As pointed out above for the pathogenic microorganisms, the mutualistic bacteria can also occupy different intracellular compartments, either the cytosol of the bacteriocytes or vacuoles. A typical example of a vacuole-residing bacterium is *Buchnera*, the primary endosymbiont of aphids, whereas the primary endosymbionts of certain ants and of tsetse flies, "*Candidatus* Blochmannia" and *Wigglesworthia*, respectively, are located in the cytosol of the insect cells (36).

EVOLUTION OF BACTERIOCYTE ENDOSYMBIONTS OF INSECTS

Transmission of the bacteriocyte endosymbionts occurs vertically, and eggs or young embryos are infected by the bacteria.

Systematic analysis has revealed that their strict vertical transmission has led to a congruent evolution of the bacteria and their host organisms. Moreover, the bacteriocyte endosymbionts described here are descendents of free-living *Enterobacteriaceae*; however, due to high substitution rates and biased nucleotide patterns, the phylogenetic relationship of these bacteria with the *Enterobacteriaceae* is still under debate (16). Interestingly, the specific living conditions of these bacteria have caused several intriguing features including the lack of most DNA repair and recombination functions but also a dramatic reduction in genome size and an extremely high AT content (70 to 80%). Several reviews of these unusual features have been published (8, 77, 80, 81, 113, 133). Due to the exclusive maternal transmission route and their obligate intracellular location, the bacteria were virtually excluded from horizontal gene transfer and recombination events with other bacteria for millions of years (50 million to 250 million years) (78, 119, 129). Moreover, due to frequent bottlenecks in their population and a resulting small effective population size, even deleterious mutations may have accumulated in these microorganisms, which in the long term may even affect their fitness and pose a threat to the symbiosis itself (62, 78, 134). In agreement with the lack of horizontal gene transfer, virtually all of the genes carried by these endosymbionts find their closest orthologs within members of the *Enterobacteriaceae*. This close relationship of the obligate intracellular bacteria with free-living members of the *Enterobacteriaceae* offers the possibility of analyzing the consequences of an obligate intracellular life-style for the metabolic properties of these bacteria, in particular a comparison of these consequences that are dependent on the various host organisms which themselves have specialized to different ecological niches.

PHYSIOLOGICAL SIGNIFICANCE OF ENDOSYMBIOTIC BACTERIA FOR THEIR HOST ORGANISMS

In many cases, the insects carrying bacteriocyte endosymbionts have specialized to a diet devoid of or scarce in certain nutrients; examples include aphids feeding on plant sap and tsetse flies feeding on mammalian blood (6, 25). While plant sap is very poor in certain nitrogen compounds, in particular in amino acids essential for the aphids (102), the blood-sucking tsetse flies make do with meals lacking several essential vitamins (86). Accordingly, the establishment of a symbiosis with bacteria may have enabled these insects to specialize to these food resources and thereby to occupy ecological niches which, without the assistance of the metabolic abilities of these bacteria, would have been impossible to colonize efficiently. In agreement with the specific nutrient composition of their diets, previous work has suggested that *Buchnera aphidicola* may provide essential amino acids to the aphid hosts whereas *Wigglesworthia glossinidia* was thought to synthesize vitamines of the B group for the tsetse flies. Several results of these early experiments have been confirmed and extended by modern genome technology (see below) (2, 112).

However, food specialization of the host insects is not obvious in all cases. For example, carpenter ants (*Camponotus* spp.) generally feed on a complex diet composed of dead and alive insects, bird excrement, and sweet food wastes. Despite their complex diet, these animals are endowed with bacterio-

cytes carrying obligate intracellular bacteria of the genus "*Candidatus* Blochmannia" (10, 104, 105, 109). On the other hand, a recent survey of ants living in tropical rain forest canopies has shown that at least in this geographical region, ants, including many *Camponotus* species, can be considered to be "secondary herbivores" since they may feed mainly on plant or insect exudates and are not predators or scavengers (24). In fact, there seems to be a general tendency in members of the genus *Camponotus* to feed on honey dew derived from sap-sucking insects, at least in certain seasons. It is possible that the endosymbiosis developed in these ants at a time where the animals were feeding mainly on such a specialized diet. In this scenario, the endosymbiotic bacteria of many "modern" *Camponotus* species with a less specialized diet may be an evolutionary relic of a former nutrient-based relationship. On the other hand, since little is known about the diet of many *Camponotus* species in nature and of seasonal changes in the food sources during the year, it is conceivable that there are ephemeral periods during which certain nutrients such as honey dew may be predominant. For survival during such periods, the animals may need the bacteria to enrich the restricted diet; concomitantly, a strong selection may favor the retention of relevant amino acid and other biosynthetic pathways in the endosymbionts. In addition to assistance in nutrient provision, the bacteria may provide other benefits for the animals. Since ants are social insects which have developed complex interaction strategies with each other and require a high hygiene standard in their nest, it is possible that the endosymbiotic bacteria are essential not only for the individual animals but also for purposes relevant at the colony level; e.g., they may contribute to the chemical language of the animals by assistance in the biosynthesis of trace pheromones or they may be engaged in the biosynthesis of antimicrobial compounds, as recently shown for a symbiosis of an extracellular actinomycete with leaf cutter ants, which protects the fungus gardens of these ants from attack by a pathogenic fungus (22).

Currently, the genome sequences of five bacteriocyte endosymbionts are available (2, 35, 112, 119, 130). These include the genomes of three *Buchnera* species resident in the aphids *Acyrthosiphum pisum*, *Baizongia pistacea*, and *Schizaphis graminum*, the genome of *Wigglesworthia glossinidia* resident in tsetse flies; and that of "*Candidatus* Blochmannia floridanus," the endosymbiont of the carpenter ant *Camponotus floridanus*. The genome sizes of these organisms vary between 615 and 705 kbp. With the exception of functions involved in translation, ribosome structure, and biogenesis, genome reduction has concerned all other functional categories currently classified in the COG database (Clusters of Orthologous Groups of Proteins; http://www.ncbi.nlm.nih.gov/COG/) (121) by comparison to the free-living *Enterobacteriaceae* such as *Escherichia coli*. In the following, we focus mainly on aspects concerning the primary metabolism of these microorganisms.

Consequences of an Obligate Intracellular Life for the Central Intermediate Metabolism

Glycolysis and citric acid cycle. Glycolysis, in which glucose is oxidized to pyruvate, is the major catabolic pathway of sugar utilization and is conserved in all kingdoms of life. This pathway is also present in *Buchnera* and "*Candidatus* Blochman-

nia." Accordingly, both organisms have a sugar-phosphotransfer import system (PTS) which may enable an efficient uptake of glucose, mannose, or related sugars and their subsequent oxidation, indicating that they take up hexoses from their host cell as an important energy and carbon source. Consistent with the presence of a PTS, no hexokinases are present in these endosymbionts. Interestingly, the *Wigglesworthia* genome encodes all glycolytic proteins but lacks the gene to encode phosphofructokinase (PfkA), which is the key enzyme in glycolysis. However, *Wigglesworthia* has retained transketolase and transaldolase of the nonoxidative branch of the pentose phosphate pathway. *Wigglesworthia* should therefore be able to oxidize hexoses to pyruvate, although without an energy yield (see below). In agreement with the lack of the glycolytic pathway and the oxidative branch of the pentose phosphate pathway (see below), it does not encode any obvious sugar uptake system. It is therefore tempting to assume that *Wigglesworthia* does not oxidize hexoses for energy generation. Instead, and in contrast to *Buchnera* and "*Candidatus* Blochmannia," the enzymes of the Embden-Meyerhoff-Parnass pathway seem to be used in gluconeogenesis rather than glycolysis, because fructose bisphosphatase (Fbp) is present, which is the key enzyme of gluconeogenesis (see below). For energy generation, *Wigglesworthia* may therefore mainly oxidize amino acids or other organic compounds derived from the host cell (Fig. 1).

All three endosymbionts encode the pyruvate dehydrogenase complex and are able to oxidize pyruvate to CO_2 and acetyl coenzyme A (acetyl-CoA), although *Wigglesworthia* appears to be the only endosymbiont which is able to synthesize CoA from panthotenate and therefore to generate acetyl-CoA without the assistance of the host cell. In contrast, *Buchnera* and "*Candidatus* Blochmannia" have to rely on their host cell for the supply of this essential coenzyme. No regular citric acid cycle is present in the endosymbionts. "*Candidatus* Blochmannia" and *Wigglesworthia* have lost the C_2-fixing steps of the citric acid cycle, while most energy-yielding reactions, i.e., those catalyzed by α-ketoglutarate dehydrogenase to fumarase, are present. Although both microorganisms have lost the malate dehydrogenase encoded by the *mdh* gene, a dissimilatory malate:quinone oxidoreductase (Mqo) is present which may participate in the cycle by production of oxaloacetate and in energy generation by feeding electrons to the ubiquinone pool of the respiratory chain. This enzyme was shown to contribute to the citric acid cycle in *E. coli*, although its role is not well understood since it cannot entirely substitute for malate dehydrogenase (128). Thus, the citric acid cycle of "*Candidatus* Blochmannia" and *Wigglesworthia* starts with α-ketoglutarate and seems to end with oxaloacetate. Consistent with the presence of a glutamate transport system, GltP, a secondary carrier, or the GltJKL ATP-binding cassette (ABC) transporter, respectively (9, 125), it is possible that transamination of glutamate to aspartate, catalyzed by AspC and using oxaloacetate as a cosubstrate, takes place, thus closing the cycle. In *Wigglesworthia* oxaloacetate can also be used for gluconeogenesis. Interestingly, in *Buchnera* the complete citric acid cycle, except α-ketoglutarate dehydrogenase, is missing (Fig. 1). α-Ketoglutarate dehydrogenase activity results in the production of succinyl-CoA, which is required for lysine biosynthesis. Since *Buchnera* encodes neither an obvious α-ketoglutarate transporter nor a transaminase to generate α-ketoglutarate from gluta-

FIG. 1. Glycolysis, TCA cycle, and gluconeogenesis in the different endosymbiotic bacteria. In *Buchnera* and "*Candidatus* Blochmannia," glucose is oxidized to acetyl-CoA, while in *Wigglesworthia*, the pathway works in the opposite, gluconeogenetic direction. In *Buchnera* the TCA cycle is reduced to α-ketoglutarate dehydrogenase activity only, while in "*Candidatus* Blochmannia" and *Wigglesworthia*, most energy-yielding steps are conserved. Transport systems for sugars and glutamate are indicated by colored circles or boxes. Features missing in the respective organism are highlighted in red. Steps between glycerol-3-phosphate and PEP are conserved in all endosymbionts and are not shown in the figure. Steps generating reductive power in the form of NADH or leading to ATP formation by substrate-level phosphorylation are indicated in green. αKG, α-ketoglutarate; Mqo, malate:quinone oxidoreductase; AspC, aspartate aminotransferase.

mate, the source of α-ketoglutarate is currently not known (see below) (Fig. 1).

Acetyl-CoA produced by the endosymbiotic bacteria should therefore be used mainly for biosynthetic processes. In fact, "*Candidatus* Blochmannia" and *Wigglesworthia* can build up fatty acids from acetyl-CoA, whereas *Buchnera* lacks the relevant enzymes (see below). *Buchnera* and *Wigglesworthia* but not "*Candidatus* Blochmannia" have retained phosphotransacetylase (Pta) and acetate kinase (AckA) and may be able to generate ATP by the production of acetate from acetyl-CoA as an additional energy supply, which may compensate to some extent for the lack of glycolysis in *Wigglesworthia* and for the missing citric acid cycle in *Buchnera* (Fig. 1).

Respiratory chain. All three endosymbionts are strictly aerobic bacteria. No genes involved in fermentative pathways could be found in either genome. As in *E. coli*, the electron transport chain consists of a primary dehydrogenase and a terminal reductase, which are linked by ubiquinone (127). "*Candidatus* Blochmannia" and *Buchnera* contain the *nuo* operon, which codes for NADH dehydrogenase I (Ndh I). This enzyme couples substrate oxidation to proton translocation by acting as a proton pump. In contrast, *Wigglesworthia* contains only the *ndh* gene, which codes for NADH dehydrogenase II (Ndh II). This enzyme does not couple substrate oxidation to proton translocation. The electrons from both NADH dehydrogenases are transferred to ubiquinone, which finally donates them to cytochrome *o* oxidase. Cytochrome *o* oxidase again acts as a proton pump, which for *Wigglesworthia* appears to be the only proton pump of the respiratory chain. All three species contain typical F_0-F_1-type ATP synthases. Figure 2 summarizes the features of the respiratory chains of the three microorganisms as deduced from their genome sequences.

Due to the lack of the tricarboxylic acid TCA cycle in *Buchnera* and glycolysis in *Wigglesworthia*, the energy yield differs strongly between the three endosymbionts, rendering "*Candidatus* Blochmannia" the fittest and *Buchnera* the least effective (Fig. 3). However, it is not known if this reflects differences in the energy requirement or supply of these bacteria. Interestingly, although a manganese-containing superoxide dismutase (SodA) is present in all endosymbionts, other detoxifying systems, including catalase, are missing. If is therefore possible that the generation of toxic oxygen species and free radicals during respiration contributes to the higher mutation rate of these bacteria than of their free-living relatives (47). Another surprising feature is the complete lack of ubiquinone biosynthetic genes in *Buchnera*, which must obtain this essential electron carrier from its host organism (112).

Pentose phosphate pathway. A major purpose of the pentose phosphate pathway is the generation of NADPH, which serves as reducing agent in many endergonic biosynthetic pathways such as fatty acid and nucleotide biosynthesis. The pathway consists of two distinct branches. In the oxidative branch, glucose-6-phosphate is oxidized and decarboxylated to ribulose-5-phosphate and NADPH is generated. First, glucose-6-phosphate dehydrogenase generates 6-phosphogluconolactone, which is converted enzymatically by 6-phosphogluconolactonase to 6-phosphogluconate, although this reaction may also occur spontaneously. Then phosphogluconate dehydrogenase further oxidizes and decarboxylates its substrate to ribulose-5-phosphate, a central building block required, e.g., for nucleo-
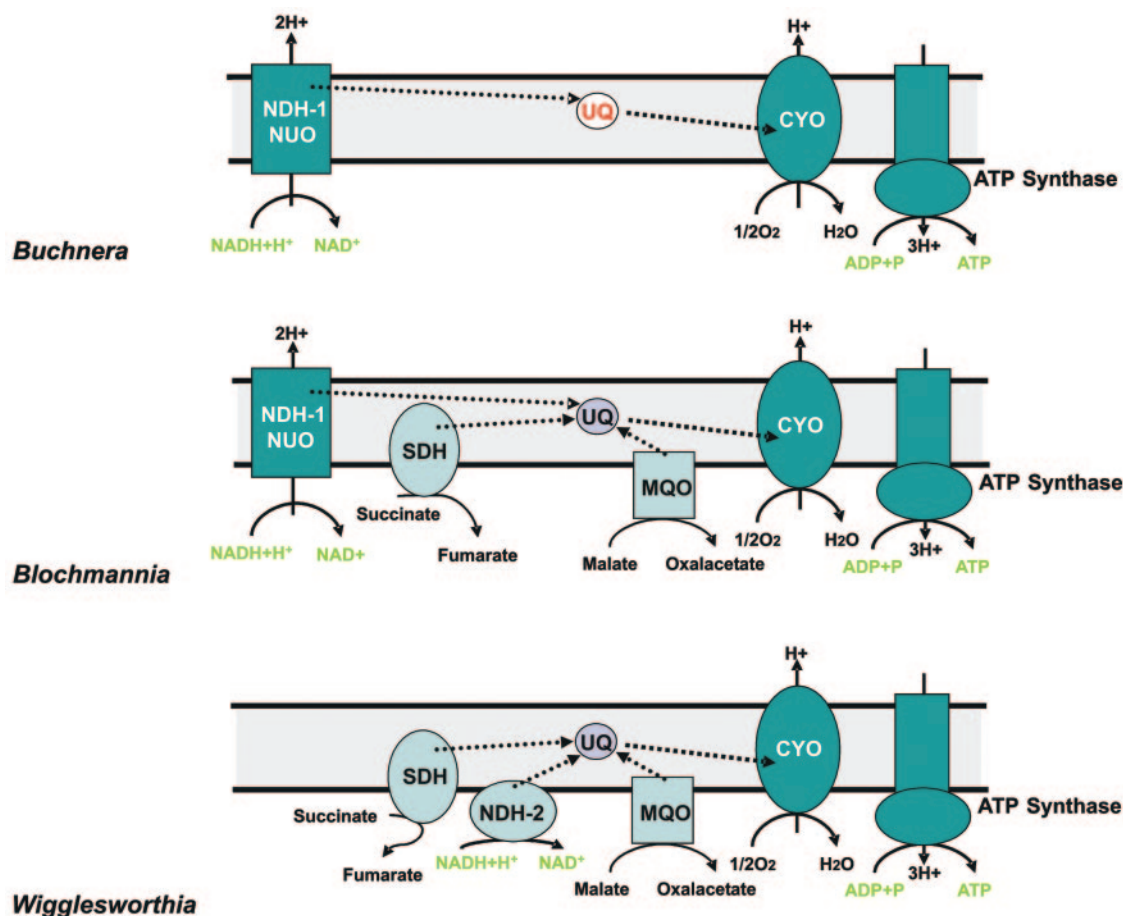
FIG. 2. Electron transport chains of the endosymbiotic bacteria. In *Buchnera*, the electron transport chain consists merely of NADH dehydrogenase I (alternative designation, NUO) and cytochrome *o* oxidase (CYO). As indicated in red, ubiquinone (UQ) cannot be synthesized by *Buchnera* but has to be provided by the host. In "*Candidatus* Blochmannia" and *Wigglesworthia*, electron transport is more complex and succinate dehydrogenase (SDH) and malate:quinone oxidoreductase (MQO) are present. In *Wigglesworthia*, NDH-1 (NUO) is replaced by NDH-2, which does not translocate protons across the membrane. Oxidoreductases coupling electron transport with proton translocation are shown in dark blue, and oxidoreductases which are not coupling are shown in light blue.

tide and cofactor biosynthesis. The nonoxidative branch of the pathway leads to the recovery of the starting substrate glucose-6-phosphate, by the concerted action of ribulose-5-phosphate epimerase and ribulose-5-phosphate isomerase as well as transketolase and transaldolase (Fig. 4).

Both *Buchnera* and "*Candidatus* Blochmannia" have a complete pentose phosphate pathway and also encode PTS sugar import systems. In contrast, and in agreement with the lack of any sugar uptake system, *Wigglesworthia* lacks the oxidative branch of the pentose phosphate pathway which enables a direct oxidation of glucose-6-phosphate, although the nonoxidative branch of the pathway is maintained in this organism (Fig. 4). *Buchnera* and "*Candidatus* Blochmannia" encode the genes for the first and third steps of the oxidative branch of the pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconatedecarboxylase, respectively. Interestingly, although the enzymatic activity of the phosphogluconolactonase (Pgl), the second enzyme of this pathway, which converts D-6-phosphoglucono-δ-lactone to 6-phosphogluconate, has been described in *E. coli* (58), no gene could be assigned to this enzyme activity. Until recently, the biological role of a 6-phosphoglu-

conolactonase was unclear, because its substrate is very unstable and subject to rapid spontaneous hydrolysis. However, the delta form, 1–5, of the lactone is the only product of glucose-6-phosphate oxidation, which by intramolecular rearrangement subsequently leads to the gamma form, 1–4. Only the delta but not the gamma form hydrolyzes spontaneously, demonstrating that the gamma form is a "dead end." Since only the delta form is a substrate for 6-phosphogluconolactonase, lactonase activity accelerates hydrolysis of the delta form, thus preventing its conversion to the useless gamma form (74).

In a review, Cordwell (18) proposed the investigation of genes of unknown function for Pgl activity, which are present in the genomic region of *E. coli* between the *modCEF* genes and the lambda attachment site, which according to classical mapping procedures should be the genome region carrying the *pgl* gene. Interestingly, of the four genes with unassigned functions in this region, only the *ybhE* gene is present in *Buchnera* and "*Candidatus* Blochmannia," whereas it is absent from the *Wigglesworthia* genome, which also lacks the other genes of the oxidative pentose phosphate pathway. It is therefore likely that the *ybhE* gene encodes the missing Pgl enzyme of the oxidate
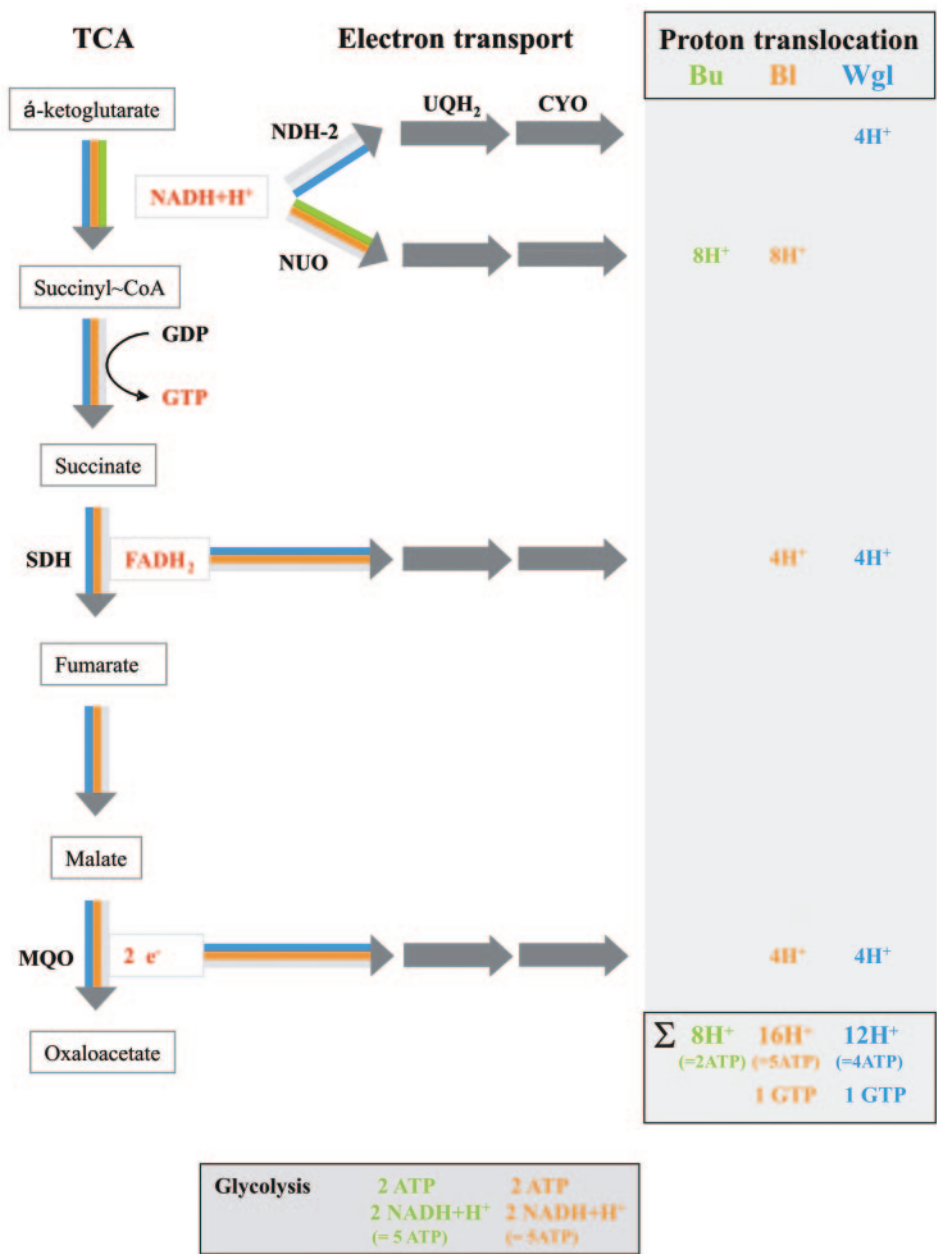
FIG. 3. Energy yield and proton translocation. *Buchnera*, "*Candidatus* Blochmannia," and *Wigglesworthia* are shown in the colors indicated in the graphic. The right panel summarizes proton translocation in the respective organisms. In *Buchnera*, a total of eight H⁺ ions are translocated, assuming that two H^+/e^- are translocated by the proton-pumping enzymes in the electron transport chain. In "*Candidatus* Blochmannia," SDH and MQO add another four H⁺ ions each to the total sum. In *Wigglesworthia*, H⁺ translocation is reduced by four H⁺ ions compared with "*Candidatus* Blochmannia," due to the non-proton-pumping NADH dehydrogenase NDH-2. ATP yield was counted on the assumption of three H⁺ ions per ATP. NDH-2, NADH ubiquinone oxidoreductase II; NUO, NADH ubiquinone oxidoreductase I (NDH-1); SDH, succinate dehydrogenase; MQO, malate:quinone oxidoreductase; CYO, cytochrome *o* oxidase.

pentose phosphate pathway. In fact, BLAST searches with YbhE reveal a weak similarity to a putative 6-phosphoglucolactonase from *Bacillus cereus*, which was assigned this function on the basis of its sequence similarity to an enzyme (Pgl) from *Pseudomonas aeruginosa* (data not shown). Reductive power required for anabolic processes in the form of NADPH can therefore be directly generated by *Buchnera* and "*Candidatus* Blochmannia" via the oxidative pentose phosphate pathway. All of these bacteria have retained a NAD kinase, and NADP

can be generated by this enzyme. In addition, a few dehydrogenases which depend on NADP for their activity are present (Table 1).

Gluconeogenesis and LPS biosynthesis. Retaining the gene for fructose bisphosphate phosphatase (*fbp*), only *Wigglesworthia* appears to be able to build up complex carbohydrates by gluconeogenesis. It is able to synthesize glucose from pyruvate, and it is the only endosymbiont encoding phosphoenolpyruvate (PEP) carboxylase, which allows gluconeogenesis starting from
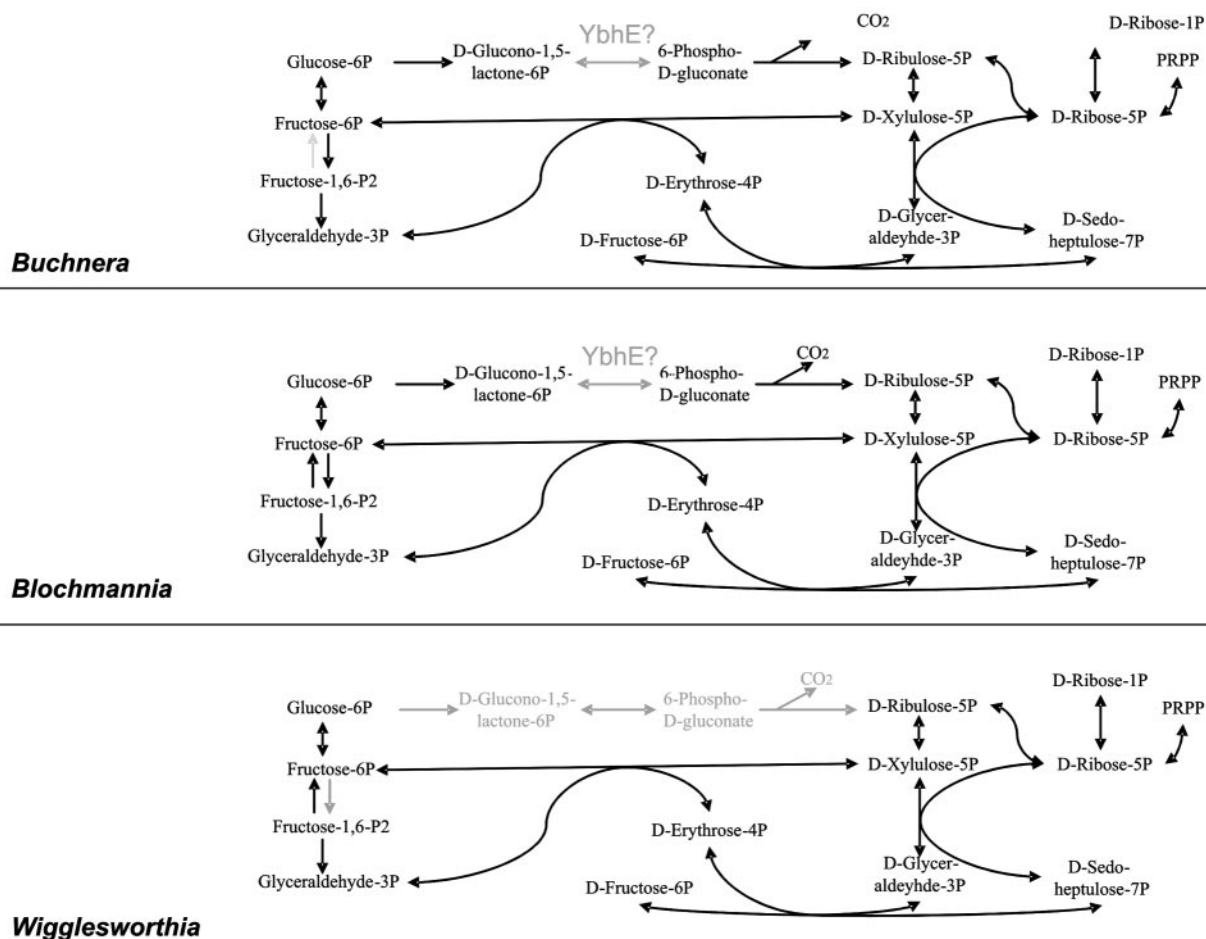
FIG. 4. Pentose phosphate pathway. In *Wigglesworthia*, the oxidative branch of the pentose phosphate pathway is missing and only the regenerative steps are present. Missing steps are shown in grey. The YbhE protein is thought to catalyze the conversion of D-glucono-1,5-lactone-6-phosphate to 6-phospho-D-gluconate (see the text for details).

oxaloacetate (Fig. 1). Oxaloacetate can be generated either from aspartate provided by the host cell through the action of aspartate transaminase AspC or by the malate-chinone oxidoreductase (Mqo) described above (Fig. 1). Gluconeogenesis is not conserved in *Buchnera* and "*Candidatus* Blochmannia" due to the deletion of the *fbp* gene.

In line with the lack of gluconeogenesis, *Buchnera* has lost nearly the entire genetic equipment required for lipopolysaccharide (LPS) biosynthesis, which in virtually all gram-negative bacteria is an essential structural feature of the outer membrane and determines many properties in their interaction with the environment. In contrast, "*Candidatus* Blochmannia" and *Wigglesworthia*, which are located in the cytoplasm, have retained several LPS biosynthetic functions. While *Wigglesworthia* should be able to build up the sugar backbone of the LPS by gluconeogenesis, "*Candidatus* Blochmannia" is endowed with a PTS and seems to rely on external sugar resources. The LPS of *Enterobacteriaceae* typically consists of three parts: lipid A, the core oligosaccharide, and the O-specific polysaccharide. In *E. coli*, lipid A biosynthesis starts with UDPGlcNac, which first undergoes a 3-O substitution and then an N substitution with β-hydroxymyristic acid. Subsequently, the diacyl derivative is dimerized and the UMP moiety

is released. Next, the 1-phospho dimer is substituted by 2-keto-3-deoxy-mannooctonic acid (KDO) derived from CMP-KDO and the hydroxyl groups of β-hydroxymyristic acid are esterified with fatty acids and phosphorylated at C-4 (94) (Fig. 5).

In *Wigglesworthia* and "*Candidatus* Blochmannia," the biosynthesis and membrane assembly of a basic LPS structure is possible. It is likely to be composed of a lipid A moiety linked to KDO (possibly KDO_2-lipid IV_A). In "*Candidatus* Blochmannia," the KDO_2-lipid IV_A moiety can be further modified by the addition of a fatty acid, probably a lauroyl residue (by analogy to *E. coli*), to the distal glucosamine unit, because it encodes the LpxL (HtrB) acyltransferase. In *E. coli* K-12, a second acyl residue consisting of a myristoyl residue is added by the LpxM acyltransferase, which is missing in "*Candidatus* Blochmannia." Interestingly, in *Wigglesworthia*, both acyltransferases, LpxL and LpxM, are missing, although in general the presence of at least one of these acyl residues in the LPS is considered essential for the viability of the bacteria. However, there are also other examples in which the genome sequences do not reveal the presence of the respective acyltransferases, indicating that some uncharacterized enzymes may have related activities (96).

TABLE 1. Dehydrogenases present in the endosymbiotic bacteria

Dehydrogenase[a]	Gene name	Present in[b]:				
		BFL	WGL	BAP	BSG	BBP
NAD-dependent dehydrogenases						
Homoserine DH	*thrA*	+	+	+	+	−
3-Isopropylmalate DH	*leuB*	+	−	+	−	+
Dihydrolipoamide DH	*lpdA*	+	+	+	+	+
Pyruvate DH E1	*aceE*	+	+	+	+	+
Pyruvate DH E2	*aceF*	+	+	−	−	+
Prephenate DH	*tyrA*	+	−	−	−	−
Succinate DH	*sdhABCD*	+	+	−	−	−
α-Ketoglutarate DH	*sucA*	+	+	+	+	+
α-Ketoglutarate DH	*sucB*	−	+	+	−	+
Glycerolaldehyde-3-phosphate DH	*gapA*	+	+	+	+	+
Glucose-6-phosphate DH	*zwf*	+	−	+	+	+
Histidinol DH	*hisD*	+	−	+	+	+
NADH DH	*nuoA-N*	+	−	+	+	+
NADH DH	*ndh*	−	+	−	−	−
Erythronate-4-phosphate DH	*pdxB*	+	+	−	−	−
4-Hydroxythreonine-4-phosphate DH	*pdxA*	−	+	−	−	−
IMP DH	*guaB*	+	+	−	−	−
Malate-chinon DH	*mqo*	+	−	−	−	−
Glycerol-3-phosphate DH	*gpsA*	+	−	−	−	−
UDP-glucose-6-phosphate DH	*ugd*	−	+	−	−	−
Proline DH	*putA*	−	+	−	−	−
UDP-*N*-acetylmuramate DH	*murB*	−	+	−	−	−
Malate DH (acceptor)	*yoiH*	−	+	−	−	−
Mannitol-1-phosphate 5-DH	*mtlD*	−	−	+	+	+
NADP-dependent dehydrogenases						
Aspartate-semialdehyde DH	*asd*	+	+	+	+	+
Shikimate-5-phosphate DH	*aroE*	+	−	+	+	+
Methylenetetrahydrofolate DH	*folD*	+	+	+	+	+
6-Phosphogluconate DH	*gnd*	+	−	+	+	+
Oxygen-dependent deyhdrogenase						
Dihydroorotate DH, cosubstrate O_2	*pyrD*	−	−	+	+	−

[a] DH, dehydrogenase.
[b] BFL, "*Candidatus* Blochmannia floridanus"; WGL, *Wigglesworthia breviplapis*; BAP, *Buchnera* from *Acyrthosiphon pisum*; BSG, *Buchnera* from *Schizaphis graminum*; BBP, *Buchnera* from *Baizongia pistacea*.

In free-living enterobacteria, the lipid A moiety is further modified by the addition of heptoses. However, the heptose biosynthesis pathway is completely missing in *Wigglesworthia* and *Buchnera*. In contrast, in "*Candidatus* Blochmannia," the heptosyl transferases WaaC (RfaC) and WaaF (RfaF) have been conserved and are involved in the modification of the LPS core with heptose. However, the heptose biosynthesis pathway which leads from sedoheptulose-7-phosphate to ADP-L-glycero-D-mannoheptose is heavily impaired, since only the HldD (RfaD) and HldE (RfaE) proteins are retained whereas the isomerase GmhA is missing and GmhB (YaeD) is a pseudogene (Fig. 6). In line with the degeneration of LPS biosynthetic enzymes, *Buchnera* does not code for the outer membrane protein Imp (for "Increased Membrane Permeability"), which was recently shown to be implicated in the transport of LPS to the cell surface and which is highly conserved in most gram-negative bacteria (11). In agreement with an apparently intact LPS core structure, the other two endosymbionts carry the *imp* gene.

In summary, *Buchnera* cannot synthesize a typical outer cell membrane whereas *Wigglesworthia* and "*Candidatus* Blochmannia" still are able to build up a core LPS structure. Why did these bacteria experience such a reduction in their LPS structure? The lipid A moiety of the LPS molecule is an extremely potent toxin and, when released from the bacteria, can cause harm to a host animal. Interestingly, *E. coli* acyltransferase mutants are viable, but pathogenic strains lacking the LpxM acyltransferase are attenuated in their virulence properties (115). Therefore, it is tempting to speculate that the stable symbiotic integration of endotoxin-carrying bacteria into a eukaryotic host has required the detoxification of this potentially dangerous compound, which, in the case of *Buchnera*, has even led to its complete removal from the bacteria. Whether the observed differences in the degree of degeneration of the LPS structure among the various endosymbiotic bacteria is due to their different locations within the eukaryotic host cell or to significant differences in the age of the respective symbioses is not known (see below). It is interesting that another intracellular bacterium of many arthropods, *Wolbachia pipientis*, belonging to the alpha-group of the *Proteobacteria*, has also lost its capacitiy to synthesize LPS (139).

Murein biosynthesis. Consistent with the reduction in the potential of these bacteria to synthesize LPS, there is also a significant slimming of the murein biosynthetic pathways; however, this appears to be quite variable in these bacteria. "*Candidatus* Blochmannia" and *Wigglesworthia* are able to syn-
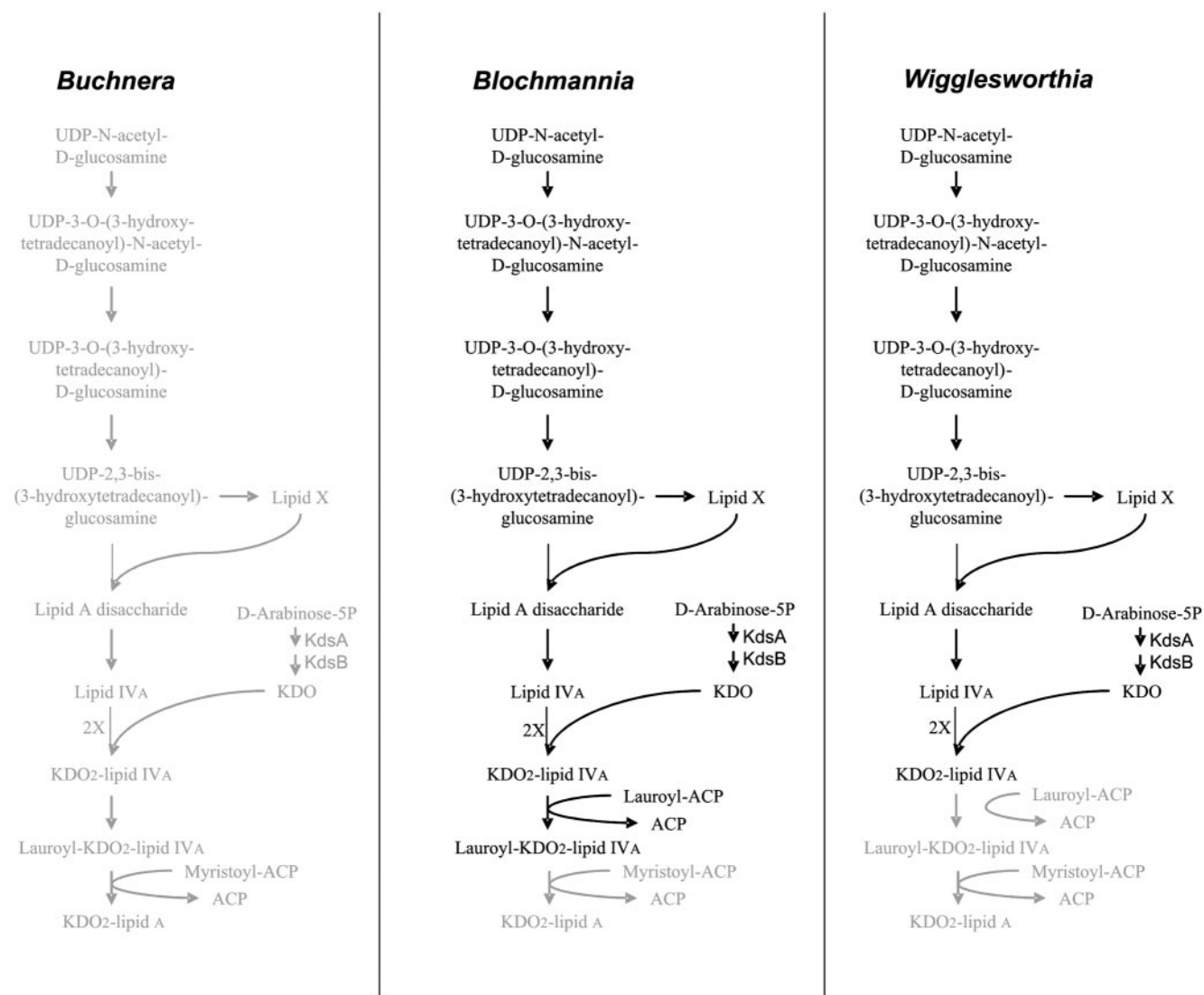
FIG. 5. Lipid A biosynthesis. Biosynthesis pathways of lipid A in the different endosymbiotic bacteria are shown. Steps missing in the respective organisms are highlighted in grey; e.g., *Buchnera* is missing the entire pathway. In contrast, in "*Candidatus* Blochmannia" and *Wigglesworthia*, only the final steps involving acylation of KDO_2-lipid IVa are partially missing.

thesize the amino sugars *N*-acetyl-D-glucosamine and *N*-acetyl-muramic acid, whereas *Buchnera* strains have lost part of this pathway (Fig. 7). However, since *N*-acetyl-D-glucosamine-1-phosphate is also produced by the host animals, the bacteria may be able to import this compound to produce the aminosugars required for murein biosynthesis. Table 2 lists proteins and enzymes involved in peptidoglycan biosynthesis present in the endosymbionts. Based on the enzyme equipment, it is likely that all endosymbionts can synthesize a peptidoglycan structure, although the conservation of various biosynthetic enzymes, such as transpeptidases and transglycosylases, and of shape-determining scaffold proteins is quite variable among them. Moreover, *Buchnera* appears to be much more impaired in its murein biosynthesis capacity, since, for example, RodA and the Mre and Mrd proteins, which are involved in the determination of bacterial shape, are missing. *E. coli rodA* mutants form round, osmotically stable cells. MreB is part of

an intracellular spiral scaffold, which assembles on the cytoplasmic face of the inner membrane. MreB mutants form spheroids or misshapen rods. In agreement with these findings, *Buchnera* but not the other endosymbionts has lost its rod-like shape and the cells are round (142).

It is interesting that, with the exception of *Wigglesworthia*, the endosymbionts lack alanine racemase. These bacteria either use the L variants of the amino acids for murein biosynthesis or are supplied with D-alanine by their host; D-alanine may be derived from the gut microflora or directly from the host. In fact, D-amino acids have been detected in peptides of various cells from animals such as amphibians, snails, crustaceans, and spiders, which are able to generate the D isomer from L-amino acids by a posttranslational reaction (57). With regard to amino acid biosynthesis, it is also interesting that *Wigglesworthia*, which has lost most of these pathways, is still able to synthesize diaminopimelic acid (DAP), which is an
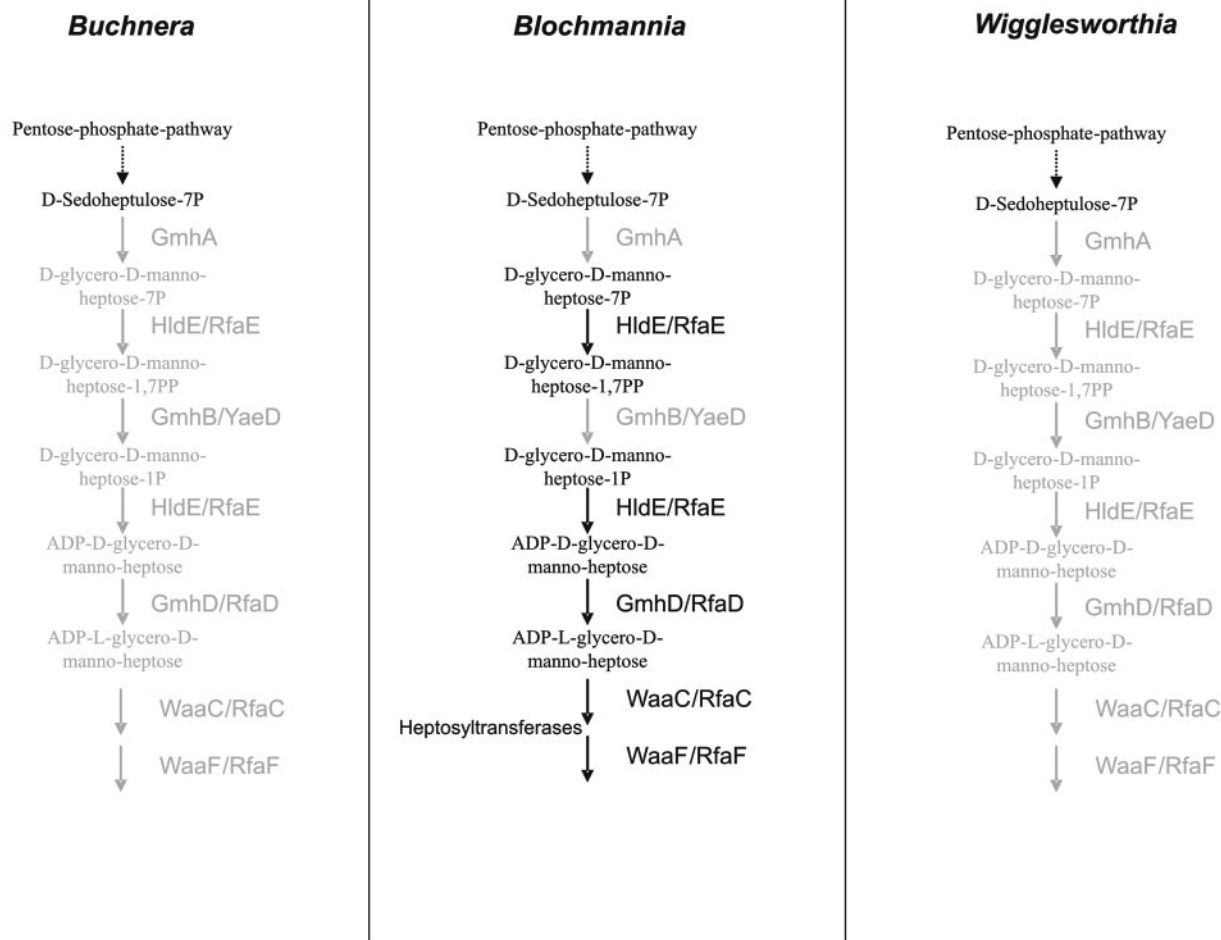
FIG. 6. Modification of LPS by heptoses. In the free-living *Enterobacteriaceae*, the LPS core is further modified with heptoses. All three endosymbionts apparently are unable to synthesize the respective heptoses. The biosynthesis and modification enzymes were entirely (*Buchnera* and *Wigglesworthia*) or partially ("*Candidatus* Blochmannia") lost, and missing steps are highlighted in grey.

intermediate of the lysine biosynthesis pathway, although lysine itself cannot be synthesized by *Wigglesworthia*. DAP, however, is an important compound in cell wall biosynthesis because it is required for the cross-linking of the peptidoglycan chains.

The prediction that these endosymbiotic bacteria are still able to synthesize a murein layer is further supported by the fact that all of them encode lipoproteins which covalently link the peptidoglycan layer with the outer membrane. In addition, lipoprotein signal peptidases and parts of the LolABCDE lipoprotein release system are present, which in *E. coli* is essential for survival (71, 84). Moreover, sequence similarities between the proteins of the LolCDE ABC transporter of *E. coli* and the hypothetical YcfUVW proteins of *Yersinia pestis* were recently noted (91). Since several of the Ycf proteins are also present in the endosymbionts, they may have taken over the function of the missing Lol proteins in the release and placement of lipoproteins. It remains curious, however, that the otherwise essential lipoprotein-specific periplasmic chaperon

LolA is missing entirely from all three sequenced *Buchnera* strains. Possibly a gene of unknown function is substituting for LolA in *Buchnera*. In contrast to free-living *Enterobacteriaceae*, the endosymbionts lack all enzymes and transport systems required for the recycling of periplasmic peptidoglycan fragments which are generated during normal growth of bacteria.

Fatty acid metabolism. In *E. coli*, fatty acid biosynthesis is carried out by a type II fatty acid synthase, a multienzyme complex encoded by the *accABCD* genes (21). Acetate residues in their activated forms as acetyl-CoA and malonyl-CoA (generated from acetyl-CoA by acetyl-CoA carboxylase AccA) are linked to the enzyme complex as thioesters. Acetate is bound to the so-called condensing enzyme and malonate is linked to the acyl carrier protein (ACP). Next, by the activity of FabD, malonate is converted to acetoacetate via elimination of CO_2 and condensation with acetate. Acetoacetate remains linked to ACP as a thioester. In the following steps, a NADPH-dependent reduction mediated by FabG, a dehydration step
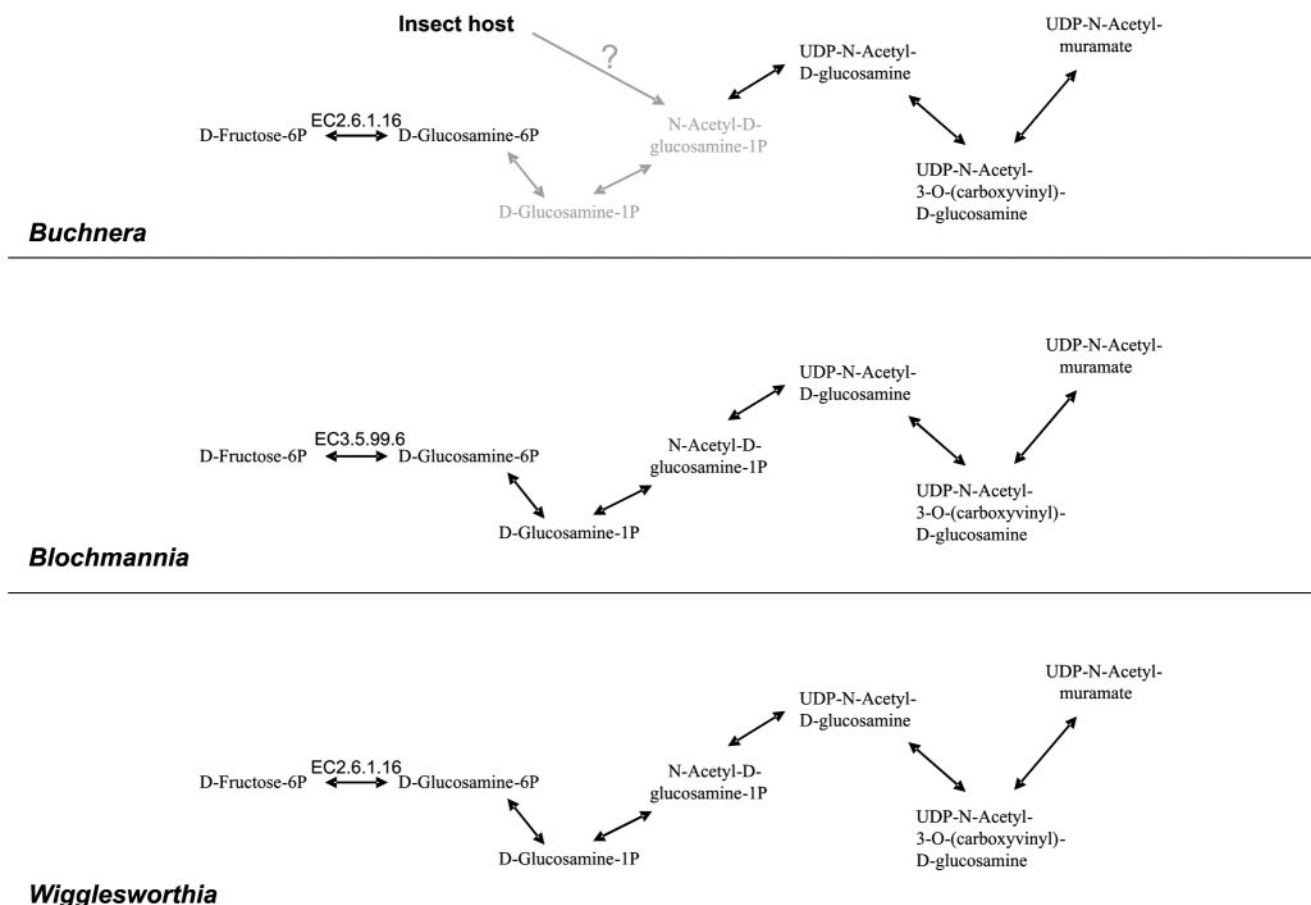
FIG. 7. Synthesis of amino sugars. "*Candidatus* Blochmannia" and *Wigglesworthia* are able to build up UDP-*N*-acetylmuramate from fructose-6-phosphate. In *Buchnera*, the transition of D-glucosamine-6-phosphate to UDP-*N*-acetylglucosamine seems to be blocked, and the missing steps are highlighted in grey. However, the lack of the respective enzymes very probably can be compensated for by provision of *N*-acetyl-D-glucosamine-1-phosphate by the host.

catalyzed by FabA and another reduction step performed by FabI follow, resulting in the production of a saturated fatty acid after several bouts of this reaction cycle.

In "*Candidatus* Blochmannia" the entire pathway is present, whereas in *Wigglesworthia* 3-oxoacyl-ACP synthase III, FabH, which catalyzes the first condensation step of acetyl-CoA with ACP, is missing. However, it is likely that this enzyme can be substituted by FabB, the 3-oxoacyl-ACP synthase I, enabling *Wigglesworthia* to perform a complete fatty acid biosynthesis. The situation in *Buchnera* is more complex, and strain-specific differences are found, although all sequenced *Buchnera* strains are probably no longer capable of fatty acid biosynthesis. All strains lack acetyl-CoA carboxylase, AccA, and FabH. In *Buchnera* strains APS but not SG or BP, FabD, which catalyzes the condensation of malonate with ACP, is also missing. Finally, FabA, catalyzing the dehydration of the growing fatty acid chain, is absent from all *Buchnera* strains, although both reductases and the acyl carrier protein are still present. In conclusion, in line with the fact that "*Candidatus* Blochmannia" and *Wigglesworthia* are both able to synthesize complex lipids such as phospholipids or LPS, they are also able to build up fatty acids from acetyl-CoA. In fact, they have retained virtually the same biosynthetic capability as *E. coli* K-12 and can synthesize saturated and unsaturated fatty acids. In contrast,

Buchnera is severely impaired in fatty acid biosynthesis. Since *Buchnera* very probably has to import phospholipids from the host organism (see below) and does not require fatty acids for LPS biosynthesis, it may not need its own fatty acid biosynthesis machinery and the respective pathways may be in the process of degeneration. Interestingly, all three endosymbionts are unable to oxidize fatty acids for energy generation, since the enzymes required for β-oxidiation are missing entirely.

Phospholipid biosynthesis. The cytoplasmic membrane of *E. coli* consists of several phospholipids, mainly phosphatidyl-ethanolamine, which makes up 70 to 80% of all phospholipids, and phosphatidylglycerol. A minor but important component is cardiolipin (21). The building blocks required for glycerolipid biosynthesis are acyl-CoA and glycerone phosphate, which is dehydrogenated to glycerol phosphate. In two consecutive steps acyl-CoA is transferred to glycerol phosphate by two different acyltransferases to yield 1,2-diacylglycerol-3-phosphate, which is subsequently activated by CTP to CDP-diacylglycerol, the major intermediate of glycerolipid metabolism. CDP-diacylglycerol can be metabolized to phosphatidyl-L-serine and decarboxylated to phosphatidylethanolamine. Phosphatidylglycerol is synthesized from glycerol-3-phosphate and CDP-diacylglycerol, which react to give phosphatidylglycerol phosphate, which is converted to phosphatidylglycerol. Cardiolipin is made from

TABLE 2. Proteins involved in cell wall biogenesis and cell division

Gene name	Function	EC no.	Present in[a]: BFL	WGL	BAP	BSG	BBP
murB	UDP-N-acetylmuramate dehydrogenase	1.1.1.158	+	+	+	+	+
murC	UDP-N-acetylmuramate-L-alanine ligase	6.3.2.8	+	+	+	−	+
murD	UDP-N-acetylmuramoylalanine-D-glutamate ligase	6.3.2.9	+	+	+	+	+
murE	UDP-N-acetylmuramoylalanyl-D-glutamate–2,6-diaminopimelate ligase	6.3.2.13	+	+	+	−	+
ddlA	D-Alanine–D-alanine ligase B	6.3.2.4	+	+	−	+	+
murF	UDP-N-acetylmuramoyl-tripeptide–D-alanyl-D-alanine ligase	6.3.2.10	+	+	+	−	+
mraY	Phospho-N-acetylmuramoylpentapeptide transferase	2.7.8.13	+	+	+	−	+
murG	UDP-N-acetylglucosamine--N-acetylmuramyl (pentapeptide) pyrophosphoryl-undecaprenol-N-acetylglucosamine transferase	2.4.1.227	+	+	+	+	+
amiB	N-Acetylmuramoyl-L-alanine amidase	3.5.1.28	+	−	+	+	+
nagA	N-Acetylglucosamine-6-phosphate deacetylase	3.5.1.25	+	−	−	−	−
nagB	Glucosamine-6-phosphate isomerase	3.5.99.6	+	−	−	−	−
glmU	UDP-N-acetylglucosamine pyrophosphorylase	2.7.7.23	+	+	+	+	+
rodA	Rod-shape-determining protein A		−	−	−	−	−
mreB	Rod-shape-determining protein MreB		+	+	−	−	−
mreC	Rod-shape-determining protein MreC		+	+	−	−	−
mreD	Rod-shape-determining protein MreD		+	+	−	−	−
mrdB	Rod-shape-determining membrane protein; cell elongation in e phase		+	+	−	−	−
mrdA	Penicillin-binding protein 2		+	+	−	−	−
mrcB	Penicillin-binding protein 1b; peptidoglycan synthetase	2.4.2-	+	+	+	−	+
uppS	Undecaprenyl pyrophosphate synthetase	2.5.1.31	+	−	+	+	+
mltA			−	−	+	+	+
mltC			−	+	−	−	−
mltE	Membrane-bound lytic murein transglycosylase E	3.2.1.-	+	−	+	−	+
dapF	Diaminopimelate epimerase		+	+	+	+	+
minC	Septum site-determining protein MinC		+	+	+	+	+
minD	Septum site-determining protein MinD		+	+	+	+	+
minE	Cell division topological specificity factor		+	+	+	+	+
ftsY	Cell division protein FtsY		+	+	+	+	+
ftsW	Cell division protein FtsW		+	+	+	+	+
ftsA	Cell division protein FtsA		+	+	+	+	+
ftsQ	Cell division protein FtsQ		+	+	−	−	−
ftsZ	Cell division protein FtsZ		+	+	+	+	+
ftsL	Cell division protein FtsL		+	+	+	+	+
ftsI	Penicillin-binding protein 3 precursor; peptidoglycan synthase	2.4.1.129	+	+	+	+	+
ftsJ	Cell division protein FtsJ		+	+	+	+	+
ftsH	Cell division protein FtsH		+	−	+	+	−
ftsK	Cell division protein FtsK		+	+	−	−	−
mesJ	Cell cycle protein MesJ		+	+	+	+	+

[a] BFL, "Candidatus Blochmannia floridanus"; WGL, Wigglesworthia breviplapis; BAP, Buchnera from Acyrthosiphon pisum; BSG, Buchnera from Schizaphis graminum; BBP, Buchnera from Baizongia pistacea.

CDP-diacylglycerol and phosphatidylglycerol by cardiolipin synthase (21) (Fig. 8).

Wigglesworthia is equipped with the full set of genes necessary for the biosynthesis of all three glycerolipids, while in "Candidatus Blochmannia" only the plsB gene, encoding the first acyltransferase is missing. Thus, both organisms are very probably able to synthesize their own membrane phospholipids. The situation is strikingly different in Buchnera. In line with the degeneration of its potential involvement in cell wall and outer membrane biosynthesis, Buchnera has lost the ability to produce phospholipids and must import either the phospholipids themselves or their biosynthetic enzymes from the host cell (Fig. 8). Therefore, although electron micrographs still show the presence of a gram-negative double membrane in Buchnera, the cell wall and outer membrane may structurally be very impaired by comparison with the apparently intact outer membrane of the cytosolic endosymbionts.

Interestingly, Buchnera has retained only one enzyme involved in phospholipid biosynthesis, cardiolipin synthase, which is also present in the other endosymbionts. In E. coli, anionic phospholipids, in particular cardiolipin, have several important functions and are involved in protein secretion (75), recruitment of the replication initiatior protein DnaA to the membrane (46), and provision of diacylglycerol moieties to outer membrane lipoproteins (118). In eukaryotic organisms, cardiolipin is found in the inner mitochondrial membrane, where it is essential for mitochondrial function (72). The conservation of cardiolipin synthase even in Buchnera, which has lost all the other phospholipid biosynthetic functions, is intriguing and may suggest that this enzyme is very important for the symbiotic organisms, although its precursors must be imported from the host. It is also possible that the bacteria provide the host cell with cardiolipin to enhance the function of the mitochondria, which are probably required by the endosymbionts to satisfy their energy demands. Activation of mitochondrial activity by an endosymbiotic bacterium, Sitophilus oryzae principal endosymbiont of weevils (S. oryzae), which is phylogenetically closely related to the endosymbionts discussed in this review has recently been described, although it was suggested that this may be achieved by the supply of vitamins such as
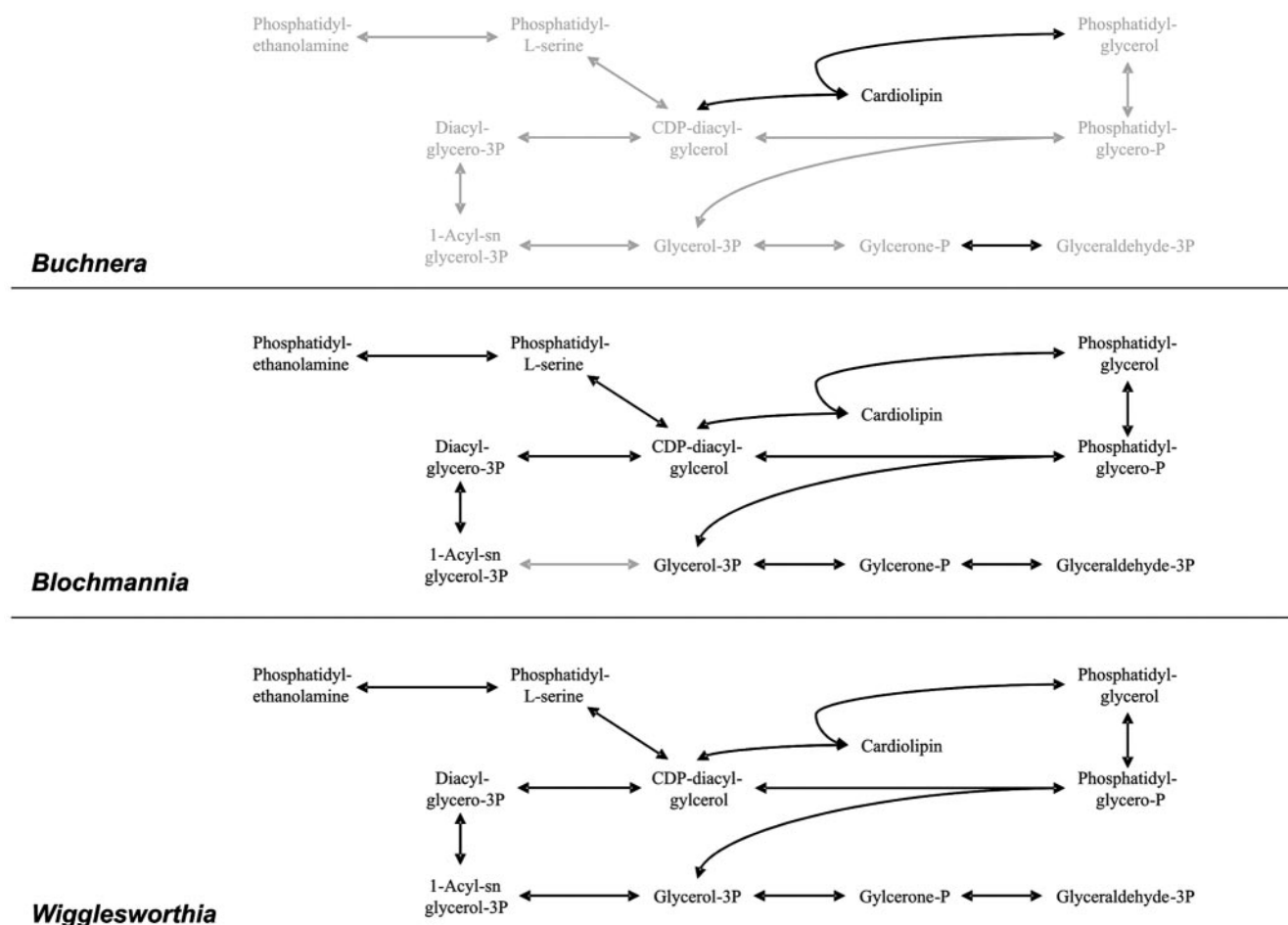
FIG. 8. Phospholipid synthesis. In *Buchnera* the complete biosynthetic pathway except cardiolipin synthase is missing. If cardiolipin synthase is still active, the respective precursors, CDP-diacylglycerol and phosphatidylglycerol, have to be provided by the host. In "*Candidatus* Blochmannia," only the glycerol-3-phosphate O-acyltransferase specific for the first acyltransfer is missing, while in *Wigglesworthia* the pathway is complete. Missing steps are highlighted in grey.

pantothenic acid and riboflavin to the host cell (41). Finally, cardiolipin is known to function as a proton reservoir in particular for bacteria living in basic habitats and may therefore have a quite general importance for proton pumping in biological membranes (52). Such adaptations may be critical, since in general the pH in the cytosol provides reducing conditions in a near-neutral environment (pH 6.8 to 7.1) whereas the extracellular environment in general has a near-neutral, slightly alcaline environment (pH 7.4).

Nucleotide metabolism. The biosynthesis of purines proceeds in a series of 10 reactions by stepwise addition of functional groups to 5-phosphoribosyl-1-diphosphate, the activated form of ribose-5-phosphate (Fig. 9). 5′-Phosphoribosyl-5-amino-4-imidazole carboxamide (AICAR) and IMP are important intermediates of the purine biosynthesis pathway. Pyrimidine biosynthesis is less complex and proceeds in three steps, with orotate formed from aspartate and carbamoyl phosphate. Orotate is then linked to 5-phosphoribosyl-1-diphosphate and decarboxylated to UMP (Fig. 10).

Purine and pyrimidine nucleotides are very abundant in the host cell, with ATP being one of the most abundant com-

pounds in the cytosol. Nevertheless, regarding nucleotide biosynthesis, the endosymbionts have retained much of their autonomy. In fact, the purine and pyrimidine biosynthesis pathways of *Wigglesworthia* are virtually identical to those of *E. coli* K-12, although in the purine pathway the *purN* gene encoding the phosphoribosylglycinamide formyltransferase responsible for formylation of 5′-phosphoribosylglycinamide (GAR) cannot be detected (Fig. 10). However, since all other enzymes appear to be well conserved, it is likely that this reaction is carried out by an alternative enzyme, leading to a complete purine biosynthesis pathway in this microorganism. Also, *Buchnera* and *Blochmannia* are expected to be able to produce purines, although in both bacteria the pathway transforming PRPP via GAR and 5′-phosphoribosylformylglycinamide (FGAM) to the purine biosynthesis intermediate AICAR was lost. However, in contrast to *Wigglesworthia*, in *Buchnera* and "*Candidatus* Blochmannia" the histidine biosynthesis pathway has been conserved (see below) and so these organisms are able to synthesize AICAR starting from PRPP via phosphoribulosyl-formimino-AICAR phosphate which is then cleaved to AICAR as an intermediate spinoff product and
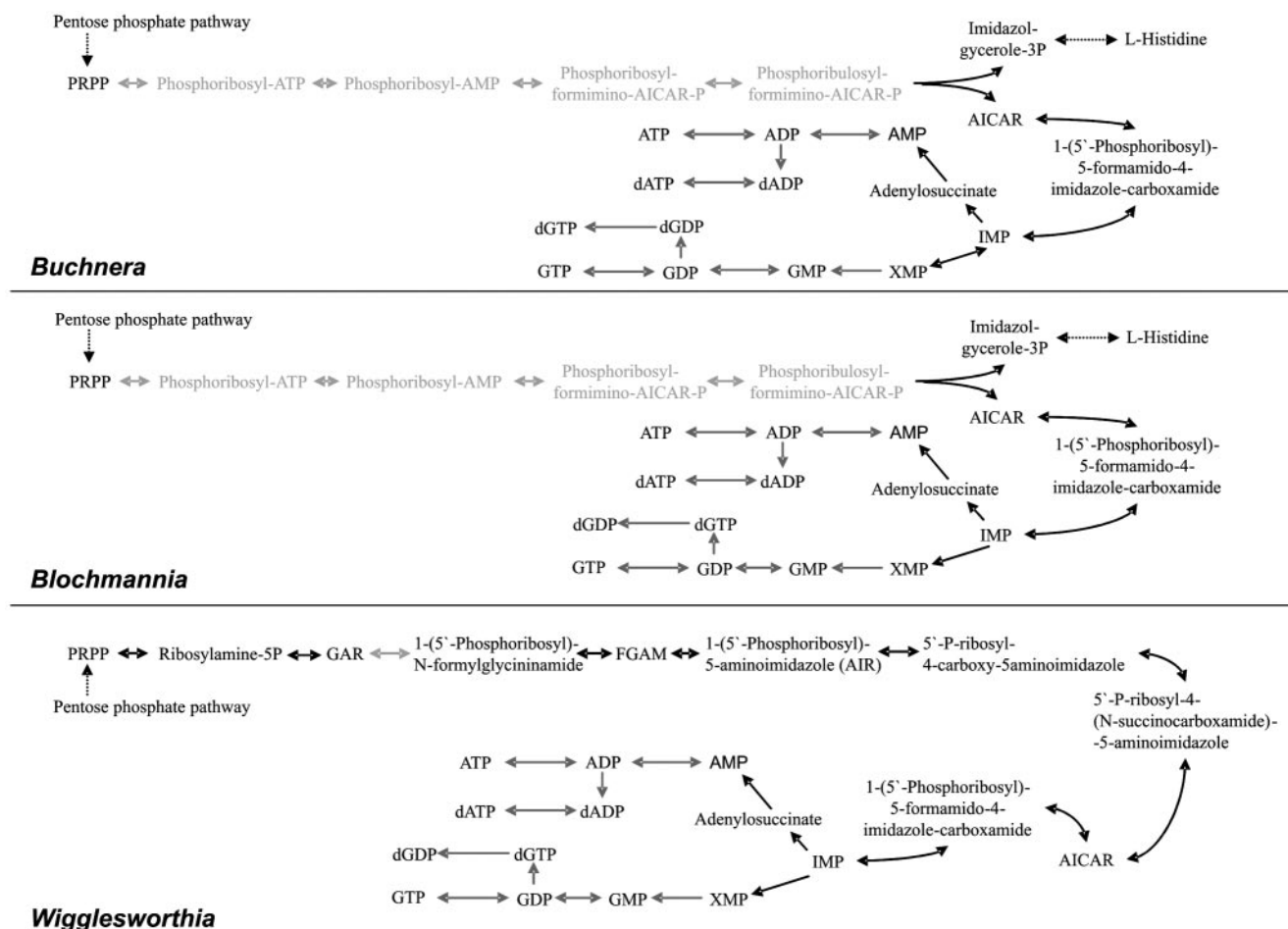
FIG. 9. Purine biosynthesis. In *Wigglesworthia*, with the exception of the phosphoribosylglycinamide formyltransferase PurN, the complete purine biosynthetic pathway is present. In *Buchnera* and "*Candidatus* Blochmannia," the first steps, leading from PRPP to AICAR, have been lost entirely. However, AICAR is also an intermediate of histidine biosynthesis in these two organisms, allowing purine biosynthesis by the combination of histidine and purine biosynthesis pathways. The conversion of purine intermediates "downstream" of the intermediates AMP, IMP, and XMP is not entirely clear from the data derived from the genome sequence, since some dedicated enzymes apparently are missing. However, as described in the text, the respective reactions are likely to be carried out by related enzymes, possibly as a result of an expansion of the substrate specificity of these enzymes. The light grey arrows highlight missing steps in purine biosynthesis, which are replaced by parts of the ehistidine biosynthesis pathuray in *Blochmannia* and *Buchnera*, and the dark grey arrows show the parts of the purine biosynthesis pathway which very probably can be carried out by the bacteria.

to imidazole-glycerol-3-phosphate, which is then further processed to generate histidine. If this kind of combination of biosynthetic pathway turns out to be operating in vivo, which in free-living bacteria such as *E. coli* are strictly separate and have their individual specific regulatory systems, this would imply that during the evolution of these endosymbiotic bacteria the regulation of such pathways may have been changed or even abolished due to a constant environment. In fact, it no longer makes sense to regulate ATP phosphoribosyltransferase of the histidine pathway by end product regulation if the same enzyme is also required for purine biosynthesis.

Not all enzymes encoded by *E. coli* required for interconversion of the nucleotides between their mono-, di-, and triphosphorylated and deoxy forms are present in the endosymbionts. However, it is likely that they can synthesize the whole set of nucleotides, since they have the capacity to synthesize all basic purine nucleotides such as IMP, AMP, and XMP (Fig. 9). To compensate for this insufficiency, there are several possibilities: (i) broader specificities of the enzymes involved, which

allow further parts of the nucleotide metabolism to occur, as exemplified in several other organisms with a reduced genome, e.g., *Mollicutes* (94); (ii) activation of salvage pathways which allow sufficient compensation in a nutrient-rich environment (possibly provided by the host organism) (110); and (iii) direct transport of nucleotides into the cytosol, similarly to several parasites. However, as mentioned above, on the basis of similarity to currently known nucleotide transporters, there are no indications in favor of this option.

Interestingly, in contrast to the other endosymbionts, "*Candidatus* Blochmannia" shows a complete degeneration of its pyrimidine biosynthesis pathway. This implies that the ant endosymbiont requires the import of pyrimidines from its host organism. In fact, the nucleoside permease NupC is present in "*Candidatus* Blochmannia." This permease may satisfy the nucleoside demands of "*Candidatus* Blochmannia," since in *E. coli* the homolog NupC has specificity toward pyrimidine nucleosides and their deoxy derivatives (Fig. 11) (20). Nucleoside transporters are apparently missing from *Buchnera* and
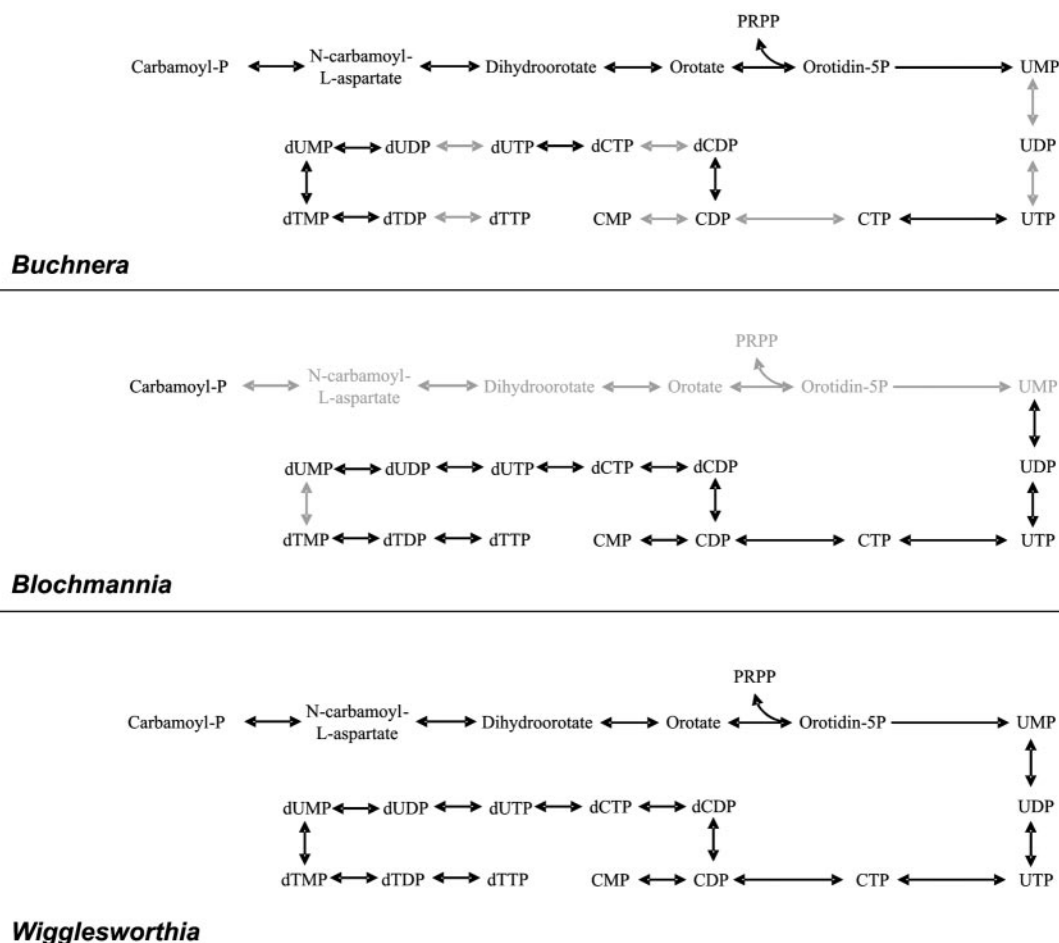
FIG. 10. Pyrimidine biosynthesis. *Buchnera* is able to synthesize UMP, but most successive steps are missing, while "*Candidatus* Blochmannia" seems to need UMP as the starting material but, with the exception of trymidylate synthase (ThyA), can catalyze all consecutive steps. Only in *Wigglesworthia* is the pyrimidine biosynthetic pathway complete. Missing steps are highlighted in grey.

Wigglesworthia. Another interesting feature of pyrimidine biosynthesis is that *Buchnera* is able to produce only the basic pyrimidine nucleotide UMP, but several subsequent steps generating cytidine and thymidine nucleotides and their deoxy variants are missing. Again, based on the mechanisms described above for the purine nucleotides, it is assumed that *Buchnera* should be able to synthesize the respective compounds.

Sulfur metabolism. Sulfate must be reduced to sulfide in order to become incorporated into biomolecules. Insects generally are not able to reduce oxidized sulfur compounds and must rely on their diet to provide them. *B. aphidicola* APS and "*Candidatus* B. floridanus" are able to reduce sulfate via the APS-PAPS pathway. In this pathway, sulfate is first reduced to sulfite. For this purpose, ATP sulfurylase forms an energy-rich anhydride, adenosine-5′-phosphosulfate (APS), followed by the formation of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), catalyzed by APS kinase. PAPS is then reduced to sulfite by PAPS reductase. Next, sulfite is reduced to H_2S by sulfite reductase under consumtion of NADPH. H_2S is immediately fixed to *O*-acetylserine, resulting in cysteine.

"*Candidatus* B. floridanus" not only is able to reduce sulfate via the APS-PAPS pathway but also has retained a sulfate-specific ABC transport system (CysAUW) (Fig. 11), which very

probably enables these bacteria to metabolize even trace amounts of sulfate (114). *Buchnera* APS is also capable of sulfate reduction, but no known sulfate carrier was identified in its genome, indicating that sulfate is taken up by an unknown transport system. *W. glossinidia*, *B. aphidicola* SGR, and *B. aphidicola* BP are not able to reduce sulfate via the APS-PAPS pathway, which indicates that their diet contains sufficient amounts of sulfur compounds to sustain their life or that sufficient amounts of reduced sulfur are provided by the gut flora.

Transport systems. (i) Small-molecule transport systems. The majority of transport systems present in the endosymbiotic bacteria is constituted by secondary carriers, in which transport activity is driven by an ion gradient across the membrane (48). Most remaining transport systems are ABC-type carriers, consisting of a membrane-spanning permease and an ATP-binding subunit which energizes transport by ATP hydrolysis (Fig. 11) (108). Interestingly, periplasmic substrate-binding proteins, which usually are an integral part of such transport systems, are missing in most ABC-type carriers of the endosymbiotic bacteria. Very few permeases catalyzing transport by a concentration gradient are found.

Only a single transport system is shared by all three endosymbionts: the secondary carrier for inorganic phosphate PitA
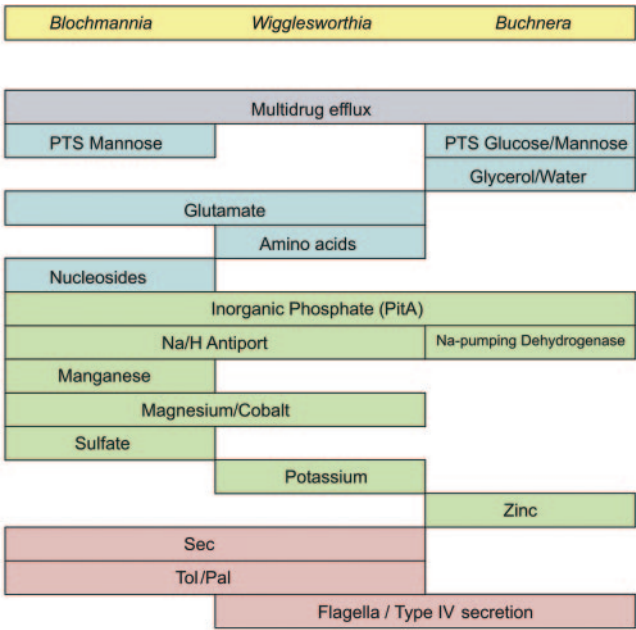
FIG. 11. Overview of the transport capacities in the different endosymbionts. In most cases, only the general transport capabilities of the endosymbiotic bacteria are shown. The dedicated transport systems may differ among the various bacteria. The *Buchnera* genome encodes the smallest number of transport systems. Colors indicate multidrug transport systems (gray), metabolite transport systems (blue), ion transport systems (green), and macromolecule transport systems (red). For details see the text.

(Fig. 11) (40). All three microorganisms also encode multidrug efflux systems with a broad substrate specificity. While "*Candidatus* Blochmannia" and *Wigglesworthia* contain EmrE, a secondary carrier which makes the bacteria resistant to a wide variety of toxic cationic hydrophobic compounds such as ethidium bromide, methyl viologen, and tetracycline, as well as intercalating dyes (67), *Wigglesworthia* and *Buchnera* share the Mdl multidrug efflux system, which is an ABC-type carrier (3). Only *Buchnera* contains the aquaglyceroporin GlpF involved in glycerol and water transport, which may also accept small uncharged organic molecules such as urea, glycine, and glycerolaldehyde as substrates (50). In accordance with the advanced degeneration of its amino acid biosynthetic capability, *Wigglesworthia* has retained several transport systems for amino acids, which are absent from "*Candidatus* Blochmannia" and *Buchnera*, e.g., BrnQ, a secondary carrier for branched amino acids, and SdaC, a secondary carrier for serine and threonine (111, 117).

"*Candidatus* Blochmannia" and *Wigglesworthia* but not *Buchnera* encode putative Na/H antiporters. In *Wigglesworthia* the NhaA Na/H antiporter is present (136), while "*Candidatus* Blochmannia" harbors a homolog of the *yjcE* gene, to which the function of a Na/H antiporter was assigned by similarity. In *Buchnera*, a sodium-dependent NADH dehydrogenase (Rfn) is present, which couples NADH oxidation to the export of sodium (39), indicating that sodium export is important for these bacteria, which may imply a detoxification function of these systems.

Surprisingly, only in *Wigglesworthia* are two different potassium transporters found, the secondary carrier Kup and the

ATP-dependent Trk system composed of several subunits. TrkA is a peripheral membrane protein bound to the cytoplasmic side of the membrane and is essential for transport activity. TrkH and TrkG are the K$^+$-translocating subunits, and TrkE seems to be involved in energy transfer (106). Interestingly, in *Wigglesworthia* only TrkA and TrkH are conserved, posing the question whether this system is functional. Potassium plays a central role in turgor maintenance in the free-living relatives of the endosymbiotic bacteria. However, since both transport systems present in *Wigglesworthia* are characterized by a low affinity for potassium and since the high-affinity Kdp transport system is not present, a function of these transport systems in turgor maintenance is uncertain.

Manganese is essential for the activity of enzymes such as oxalate oxidase and glutamine synthetase. Manganese-containing superoxide dismutase is the principal antioxidant enzyme of mitochondria. A number of manganese-activated enzymes play important roles in the metabolism of carbohydrates and amino acids (64). Manganese-containing enzymes such as pyruvate carboxylase and PEP carboxykinase play important roles in gluconeogenesis, and arginases required for the urea cycle also contain manganese. Despite the obvious importance of manganese for all living cells, only "*Candidatus* Blochmannia" encodes a dedicated manganese carrier, MntH (68). Additionally, the bf140 and bf141 gene products may constitute an ABC transport system which has significant similarity to other manganese carriers, so that there may be two manganese transport systems in "*Candidatus* Blochmannia." Since manganese is required for many enzyme activities, it is expected that the other endosymbionts import manganese via other systems that have not yet been identified. Both "*Candidatus* Blochmannia" and *Wigglesworthia* contain CorA, a permease specific for magnesium and cobalt (53). In addition, "*Candidatus* Blochmannia" encodes a putative cobalt efflux carrier, CorC, which also has a distinct similarity to hemolysin-related proteins (90, 95). Finally, an ABC carrier for zinc, the ZnuAB system (89), is present in *Buchnera.* Only very few carriers of unknown function are present in the endosymbiotic genomes.

In agreement with the minimal genomes present in these bacteria, only minor transport capacity was retained in their genomes. This is somewhat surprising, since one would expect massive metabolite fluxes between the symbionts and their host cells and therefore a large number of transport systems, as is the case, for example, in parasitic and symbiotic bacteria such as *Chlamydia* and *Bacteroides*, respectively (51, 140). Interestingly, *Wolbachia pipientis*, a frequent obligate intracellular parasitic companion of many arthropods belonging to the alpha-*Proteobacteria*, also has a reduced genome of 1.27 Mb and encodes only a very limited number of transport systems (139). The small number of transport systems might therefore allow conclusions about the importance of the transported substrate for the metabolism of the bacteria: the PTS systems of *Buchnera* and "*Candidatus* Blochmannia," which both are able to oxidize glucose by glycolyis, the glutamate transporters of "*Candidatus* Blochmannia" and *Wigglesworthia*, which both seem to feed glutamate into their truncated TCA cycle, and the transport systems for sulfate and pyrimidine nucleotides in "*Candidatus* Blochmannia," which have already been described in various sections of this review. It is also worth mentioning that *Buchnera* encodes a much smaller number of

transport systems than the other two species (Fig. 11), which may either be due to the longer evolutionary history of this symbiosis or be due to its close association with the host-derived vesicle membrane.

(ii) Transport of macromolecules. The Sec protein export system enables protein translocation across the inner membrane into the periplasmic space or the integration of proteins in the cytoplasmic membrane. It consists of SecB, a chaperone which guides the proteins to the exit place, SecA, a peripheral membrane protein with ATPase activity, the SecYEG translocase complex, and the SecDF accessory proteins. The SecDF and SecG proteins are not essential for protein export (27). In the endosymbiotic genomes, the Sec system is conserved to different degrees (Fig. 11). In *Buchnera*, all components except the nonessential *secDF* genes are present, strongly arguing for a functional Sec protein export system in this bacterium. In "Candidatus Blochmannia," most *sec* genes are conserved except for *secG*, which is nonessential, and *secB*, which may be functionally replaced by a different chaperone. Thus, there also seems to be a functional Sec protein export system in "Candidatus Blochmannia." Similarly, in *Wigglesworthia*, the *secA*, *secE*, *secF*, and *secG* genes are conserved, indicating a functional Sec protein export system.

In "Candidatus Blochmannia" and *Wigglesworthia*, the Tol-Pal system, consisting of the *tolQRAB*, *pal*, and *ybgF* genes, is present (Fig. 11). This system forms a protein complex which spans the periplasm and has components in the inner and outer membrane. Tol-Pal systems confer outer membrane stability and are also involved in the translocation of group A colicins and other macromolecules across the cell envelope (63, 66). Recently it was observed that *tol-oprL* mutants of *Pseudomonas putida* are impaired in growth with glycerol, fructose, and arginine as a result of a reduced transport capacity of the respective carbon source (65). From these findings, it was concluded that Tol-Pal systems are also required for the proper functioning of certain transport systems. *Buchnera* does not encode Tol-Pal-related functions. Since *Buchnera* has a strongly reduced cell wall and is tightly surrounded by a host cell-derived membrane, this different environment may have made the Tol-Pal system dispensable for *Buchnera*, whereas the cytosolic "Candidatus Blochmannia" and *Wigglesworthia* still require this system to stabilize their membranes and cell wall.

Despite their spatially restricted habitat within host cells, *Buchnera* and *Wigglesworthia* contain an almost complete flagellar machinery. It is possible that during certain developmental stages of the host the bacteria are motile, leave the bacteriocytes, and move to different tissues, such as the ovaries of their host. On the other hand, it may well be that these proteins serve as type IV secretion systems and are involved in the exchange of proteins or other macromolecules with the host cell. Protein secretion via a related system has recently been described for *Yersinia enterocolitica* (141).

Specific Metabolic Adaptations of Bacteriocyte Endosymbionts of Different Insects

Biosynthesis of essential amino acids by the aphid endosymbiont *Buchnera*. As already mentioned for the endosymbiosis of *Buchnera* with aphids, previous work had suggested that these bacteria supply nutrients such as essential amino acids to

their host insects. This is necessary because these insects thrive on a very unbalanced diet that has an excess of carbohydrates relative to nitrogen compounds including certain amino acids (25, 102, 103). Insects apparently require 10 essential amino acids which are particularly rare in plant sap. Although difficult to interpret due to side effects and negative effects on other resident bacteria in the animals, previous attempts to cure the insects of their primary endosymbionts (resulting in aposymbiotic animals), for example by adding antibiotics to the diet, indicated the absolute requirement of these bacteria for the development and survival of the aphids (137). Various metabolic studies which compared symbiotic aphids with aposymbiotic animals indicated that at least tryptophan, valine, leucine, and phenylalanine are provided by the symbiotic bacteria (26, 27a). The genome sequences of these organisms now show that they have in fact retained the biosynthesis pathways for essential amino acids required to enrich the respective diet of their host animals (112, 119, 130). As shown in Table 3, with the exception of methionine, *B. aphidicola* APS has retained the biosynthetic pathways of all amino acids essential to insects such as arginine, valine, leucine, isoleucine, lysine, threonine, histidine, phenylalanine, and tryptophan. Instead, only very few nonessential amino acids can be synthesized by the bacteria. Depending on the host organism, the degeneration of the amino acid biosynthetic potential of *Buchnera* species may still be an ongoing process, since in *B. aphidicola* BP and SG even the cysteine biosynthesis pathway was lost. *Buchnera* strains have also lost the ability to synthesize the precursors of some essential amino acids. For example, glutamate and aspartate have to be imported from the host prior to biosynthesis of the respective essential amino acids, although no respective transport system has been identified so far in *Buchnera*. This is a striking example of the mutual interdependence of the metabolic activities of the host and its endosymbiont (112, 143). Interestingly, glutamate is the major nitrogen component of phloem sap (102), in agreement with the lack of the respective biosynthetic capability in *Buchnera*. This conservation of amino acid biosynthetic genes by the various *Buchnera* strains is in marked contrast to what is observed in several parasitic bacteria including *Chlamydia trachomatis*, *Borrelia burgdorferi*, *Mycoplasma genitalium*, and *Rickettsia prowazekii*, which have lost their capacity to synthesize amino acids (4, 31, 32, 116). Although earlier work indicated that ammonium ions may be utilized by *Buchnera* as a nitrogen source (25), the genome sequences show that *Buchnera* lacks crucial enzymes required for assimilation of nitrogen such as the glutamine synthetase and a glutamate synthase, indicating a strict dependency of these bacteria on amino acid-derived nitrogen.

Several plasmids which harbor amino acid biosynthetic genes have been identified in *Buchnera* spp. (12, 60, 131). There are plasmids encoding a putative anthranilate synthase (TrpEG), which catalyzes the first step of tryptophan biosynthesis. Other plasmids contain genes required for leucine biosynthesis (*leuA*, *leuB*, *leuC*, and *leuD*). Plasmid-mediated amplification of biosynthetic genes appears to be a more common phenomenon and has also been reported for other bacteria such as cyanobacteria, where genes required for cysteine biosynthesis can be plasmid located (85), or for *Vibrio anguillarum* and its histamine biosynthesis genes (5). Gene amplification by transfer to plasmids with a high copy number may be a means

TABLE 3. Amino acid and cofactor biosynthetic capability of the endosymbiotic bacteria[a]

Amino acid or cofactor	Present in[b]:				
	BAP	BSG	BBP	WGL	BFL
Essential amino acids					
Arginine	+[c]	+[c]	+[c]	−	−
Valine	+	+	+	−	+
Leucine	+	+	+	−	+
Isoleucine	+[d]	+[d]	+[d]	−	+
Lysine	+	+	+	−[e]	+
Threonine	+	+	+	−[f]	+
Methionine	−	−	−	−	+
Histidine	+	+	+	−	+
Phenylalanine	+	+	+	−	+
Tryptophan	+	+	+	−	+
Nonessential amino acids					
Tyrosine	−	−	−	−	+
Cysteine	+	+	−	−	+
Glycine	+	+	+	+	+
Serine	−	−	−	−	−
Proline	−	−	−	−	−
Glutamine	−	−	−	+	+
Glutamate	−	−	−	+	+
Aspartate	−	−	−	+	+
Asparagine	−	−	−	−	−
Alanine	−	−	−	−	−
Cofactors					
Biotin	−	−	+	+	−
Lipoic acid	+	+	+	+	−
FAD	+	+	−[g]	+	+
Folate	−[h]	−[h]	−[h]	+	+
Pantothenate	−	−	−	+[i]	−
Thiamine	−	−	−	+	−
Pyridoxine	−	−	−	+[j]	+[j,k]
Pyridoxine phosphate	−	−	−	+[k]	+[k]
Protoheme	−	−	−	+	−
Ubiquinone	−	−	−	+	+
NAD	+[l]	+[l]	−	+[m]	−

[a] Adapted from reference 35 with permission of the publisher.
[b] BAP, *Buchnera aphidicola* from *Acyrthosiphon pisum*; BSG, *Buchnera aphidicola* from *Schizaphis graminum*; BBP, *Buchnera aphidicola* from *Baizongia pistacea*; Wgl, *Wigglesworthia glossinidia*; BFL, "*Candidatus* Blochmannia floridanus."
[c] Synthesized from ornithine.
[d] Synthesized from α-ketobutyrate and pyruvate.
[e] The pathway ends with the synthesis of diaminopimelate.
[f] The pathway ends with the synthesis of homoserine.
[g] The pathway ends with the synthesis of riboflavin.
[h] Synthesized from 7,8-dihydropteroate.
[i] Synthesized from 3-methyl-2-oxobutanoate.
[j] Synthesized from pyridoxamine.
[k] Synthesized from d-erythrose-4-phosphate.
[l] Synthesized from nicotinate.
[m] Synthesized from quinolinate.

symbionts of several aphids of the genus *Diuraphis*, it seems that pseudogenes are an ancient and universal feature of these bacteria and have arisen independently in several *Diuraphis* lineages (135). The assumption that amplification and subsequent silencing of amino acid biosynthesis genes is merely a direct reaction to the availability of the essential amino acid in the plant sap is therefore probably too simplistic, although it may explain this phenomenon to some extent. However, silencing or reduction activities may help in controlling the growth of *Buchnera* or might be involved in reaching an adequate metabolic flux balance between the different pathways of the host and *Buchnera*. In fact, recent data indicate that the genome of *Buchnera* is highly polyploid and may be present at more than 100 copies per cell. Therefore, it is also possible that the bacteria lacking virtually all regulatory factors involved in the regulation of amino acid biosynthesis regulate the expression of certain amino acid pathways via the copy number of the respective plasmids (54, 82, 93).

Cofactor biosynthesis by the tsetse fly endosymbiont *Wigglesworthia* as a possible key for its symbiotic function. The gene content related to specific functions of the symbiosis is quite different in *Wigglesworthia*, the endosymbiont of tsetse flies. *Wigglesworthia* has retained many biosynthetic pathways required for cofactor and vitamine biosynthesis. There are about 62 genes involved in the biosynthesis of cofactors, prosthetic groups, and carriers. According to the genome sequence, *Wigglesworthia* is able to synthesize pantothenate, biotin, thiazole, thiamine, flavin adenine dinucleotide, lipoic acid, pyridoxine, protoheme, nicotinamide, and folate (2). The conservation of these biosynthetic pathways fits well with the fact that mammalian blood is quite poor in certain cofactors and vitamins, in particular vitamins of the B complex. The genome sequence nicely confirms previous experiments which already indicated that *Wigglesworthia* might be implicated in providing the flies, in particular, with vitamins of the B complex (86). In contrast to *Buchnera, Wigglesworthia* has lost most of the amino acid biosynthetic pathways. It encodes factors engaged in a few steps involved in the biosynthesis of the nonessential amino acids glycine, glutamate, glutamine, aspartate, and DAP. Accordingly, although *Wigglesworthia* encodes only very few transport systems, several of them apparently are involved in amino acid import (see above).

Metabolic interactions in the ant-"*Candidatus* Blochmannia" symbiosis. Although ants of the genus *Camponotus* in general are omnivorous animals, they show a preference for honey dew and other sweet secretions from plants and animals, as well as for urea from animal exudates. The genome sequence of "*Candidatus* Blochmannia" indicates that this symbiosis also has a nutritional basis, with the bacteria having retained almost all biosynthetic pathways for amino acids which are essential for the host, with only the arginine biosynthetic pathway missing. The biosynthetic capability for nonessential amino acids, on the other hand, is largely reduced, and the most remarkable feature is the presence of tyrosine synthesis (Table 3). Holometabolous insects need large amounts of aromatic amino acids such as tyrosine for the sclerotization and melanization of their cuticle during ecdysis, and it is likely that the bacteria contribute significantly to satisfy this demand. Since the conservation of entire biosynthetic pathways, despite the extreme genome reduction in these bacteria, may be indic-

of adaptation to specific new environments which may require high-level expression of certain gene products (100). Surprisingly, some plasmids of *Buchnera* that carry *trpEG* contain mutations which should lead to a silencing of the expression level of anthranilate synthase, e.g., by mutations in the presumptive promoter regions or by pseudogene formation (7, 61, 131). The biological significance of gene amplification and silencing for *Buchnera* and the evolutionary forces leading to these events are not clear yet. As expected from their important function for host nutrition, it should be anticipated that the amino acid biosynthetic genes are subject to a strong host-level selection for their functionality; however, for the endo-

ative of an important role of the respective pathway for the symbiosis, it is tempting to speculate that bacterial tyrosine biosynthesis may have a prominent function for the ants. In accordance with the preference of the host for a diet rich in urea (N. Blüthgen, personal communication), a complete urease gene cluster is present in the bacterial genome. Urease hydrolyzes urea to produce CO_2 and ammonia, the latter of which can be fed into amino acid metabolism by the activity of glutamine synthetase, which is also encoded by "*Candidatus* Blochmannia." Another striking feature of "*Candidatus* Blochmannia" is the lack of arginine synthesis, although all other essential amino acids can be synthesized. This indicates that arginine is not limiting in this system and is degraded rather than synthesized. Arginine is an amino acid which is particularly rich in nitrogen and could serve as a nitrogen storage compound. It can be cleaved into ornithine and urea by arginases of the animal host or by a bacterial protein (Bf1253) of the arginase family. Thus, arginine could serve as a nitrogen store to keep amino acid synthesis running in times of high metabolic activity but no food uptake, e.g., during pupation.

Only two enzymes of the arginine synthesis pathway, carbamoyl-phosphate synthase (CarAB) and ornithin carbamoyl-transferase (ArgI), are retained in "*Candidatus* Blochmannia," enabling the bacteria to synthesize citrulline from ornithine. This includes the possibility that the endosymbionts take part in a urea cycle similar to that known of mammals, where the corresponding part of the urea cycle is localized in the mitochondria. However, this urea cycle would short-circuit the arginine-urea pathway suggested above. Therefore, if both reaction pathways are relevant to this symbiosis, they are likely to be operating during different stages of the life of the animal.

"*Candidatus* Blochmannia" has retained the glycolytic pathway and is able to synthesize acetyl-CoA from the oxidation of pyruvate. However, unlike the other endosymbionts, it is not able to synthesize acetate from acetyl-CoA and thus gain ATP. The only way to dispose of acetyl-CoA and recover Co A is to feed acetyl-CoA into fatty acid synthesis. Thus, "*Candidatus* Blochmannia" may supply its host not only with essential amino acids but also with fatty acids.

GENERAL CONCLUSIONS

Common Themes in the Metabolic Activities of Bacteriocyte Endosymbionts

An obvious common theme observable in all endosymbiotic genomes is the selective elimination of biosynthetic pathways which are redundant in the respective habitat. In fact, there are several examples, which indicate that the bacteria have to obtain the respective compounds or their biosynthetic machinery from the host. For example, *Buchnera* and "*Candidatus* Blochmannia" have reduced synthesizing capacity for coenzymes and *Buchnera* has also lost the capacity to synthesize aminosugars, complex carbohydrates, fatty acids, and phospholipids. "*Candidatus* Blochmannia" has lost the capacity to synthesize pyrimidines. In general, the pathways which are retained are those which apparently lead to compounds which the host cannot supply or for which the host is auxotrophic, such as essential amino acids in the case of aphids and ants or of vitamins and cofactors in the case of *Wigglesworthia*, which are not present in

their diet in sufficient amounts. In some cases, existing pathways are combined when they have common intermediate products, as exemplified by the biosynthesis pathways of purine nucleotides and histidine in *Buchnera* and "*Candidatus* Blochmannia."

Essential metabolic pathways are reduced to a minimum. All of the insect endosymbionts have a strict respiratory metabolism, reflecting the very stable habitat. Of the 15 primary dehydrogenases and 10 terminal reductases present in the very closely related free-living *E. coli* (127), only NADH dehydrogenase (Ndh I or Ndh II) and cytochrome *o* oxidase are retained in the endosymbionts. Not only was energy metabolism adapted to life under conditions of high oxygen pressure, but also isoenzymes, catalyzing the same reactions were abolished. The citric acid cycle was reduced partially (in *Wigglesworthia* and "*Candidatus* Blochmannia" or entirely (in *Buchnera*). All endosymbionts have strongly reduced anabolic and catabolic capacitites, indicating a very high degree of specialization to a particular and probably very stable intracellular habitat. This specialization is also reflected in the nearly complete lack of regulatory factors and signal transduction systems.

A second feature which turns out to hold true for all of these endosymbiotic associations is the mutual interdependence of the metabolism of the host and its symbiont. Precursors have to be delivered by the host, some of them are used by the endosymbiont and some of them serve as substrates to synthesize symbiosis-relevant metabolites for the host. *Wigglesworthia* synthesizes vitamins and cofactors for its host but has to be supplied with amino acids. *Buchnera* and "*Candidatus* Blochmannia," on the other hand, need their host to supply them with vitamins and other metabolites to enable their synthesis of essential amino acids for the host.

Mechanisms of Metabolic Pathway Evolution

Evolution of enzymes and pathways in endosymbionts follows specific rules. These are summarized after a short overview of general evolution of pathways. Figure 12 gives an overview of the different mechanisms of pathway evolution.

Recent studies show that recruitment of single enzymes from different pathways seems to be the driving force for pathway evolution (107). Other mechanisms such as pathway duplication, enzyme specialization, de novo invention of pathways, and retroevolution of pathways seem to be less abundant. In enzyme superfamilies, about 20% turn out to be quite variable not only in changing reaction chemistry or metabolite type but even both at the same time (107). These variable superfamilies account for nearly half of all known reactions. The most commonly occurring metabolites provide a helping hand for such changes since they can be accommodated by many enzyme superfamilies. New or modified pathways thus prefer to evolve around central metabolites, thereby keeping the overall topology of the metabolic network.

Regarding endosymbionts, their most ancient pathways evolved without adopting existing enzymes; e.g., the different kinds of tRNA synthetases evolved independently and only then became involved in different pathways such as protein translation, tRNA-dependent transamidation, and nondiscriminating acylation (76). Thereafter, endosymbionts developed very few new species-specific enzymes or folds. For example, in
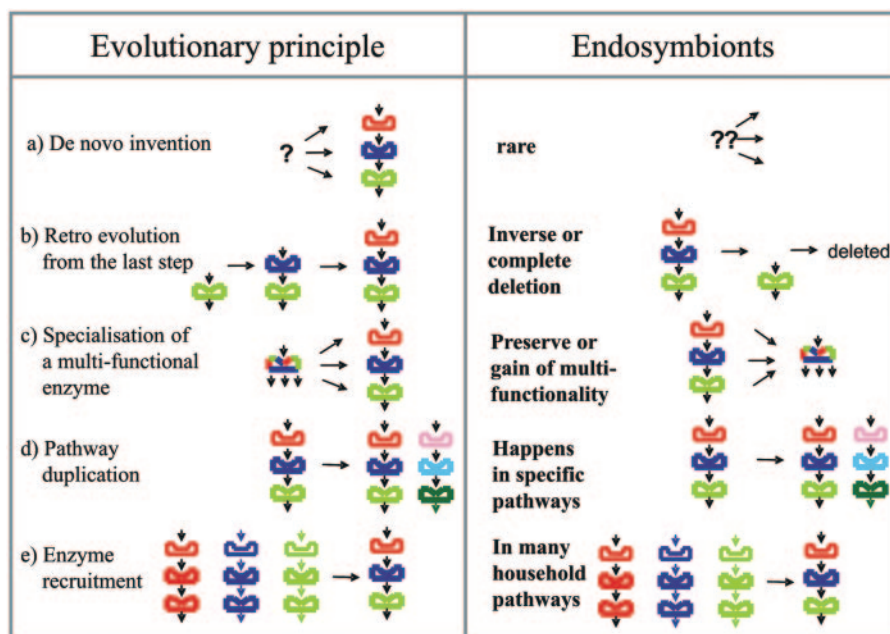
FIG. 12. Models of pathway evolution in endosymbionts. Several theories of pathway evolution (left side) and their endosymbiontic modification (right side) are shown. Pathways may have evolved (bold arrows) in different ways: (a) de novo (all reactions evolved independently from an unknown origin), (b) backwards (retroevolution), (c) by specialization of a multifunctional enzyme (left side, with multiple substrates in blue), (d) duplication of the complete pathway, or (e) by recruitment of enzymes from different pathways. The scenarios shown on the right evolve with specific modifications in endosymbionts; selection pressure is sometimes in the opposite direction, pathway reductions occur to a large extent, and selected pathways evolve, e.g., to provide metabolites useful for the host.

"*Candidatus* B. floridanus" the proteins with unknown functions were all annotated in the original publication on the genome sequence to be conserved in other (mostly gram-negative enterobacterial) species, except for "*Candidatus* B. floridanus" 390. However, meanwhile it was found that according to the classification of the COG database, this protein belongs to the COG3710 family, containing a DNA-binding winged helix-turn-helix domain (P. Gaudermann, M. Gross, and T. Dandehar, unpublished data).

Retroevolution of pathways proposes that the selective pressure on a pathway targets mainly the successful production of its end product (43). The formation of the required end product from an intermediate metabolite increases the fitness of the organism. As the end product can be derived from more and more "distant" metabolites, fitness increases and the pathway evolves backwards. This retroevolution has been proposed for the evolution of the glycolytic (30) and mandelate (92) pathways. In endosymbionts, selection often operates in the opposite direction. There is a reduction of the whole genome as more and more activities are maintained by the host with a fitness increase as long as the symbiosis prospers. Only the late steps of a metabolic pathway are then maintained. This is the case, for instance, for parts of the amino acid metabolism in endosymbionts. However, in several cases the reduction of the metabolic activities of the endosymbionts goes as far as to delete the whole pathway. Pathways can also evolve from multifunctional enzymes (101). Starting from a multifunctional enzyme catalyzing consecutive steps, the pathway might have evolved by duplication and diversification of this precursor enzyme to the more specific and efficient enzymes known today, which catalyze only one step each in the pathway. O'Brien

and Herschlag (87) analyzed several enzymes with alternative reactions distinct from their normal biological reaction to support the concept that broader substrates and reaction specificities are subsequently captured by adaptive evolution, such as for carbamoyl phosphate synthase and β-D-glucan hydrolases in higher plants (44). In endosymbionts and other compact genomes, there is, however, a certain selection pressure to select or at least preserve enzymes with a broader specificity to allow several reactions to be catalyzed just by one enzyme; an example is that of different metabolic kinases (94). We think that this may extend to some of the regulatory enzymes in "*Candidatus* B. floridanus" and very probably in the other endosymbionts. Recent data obtained with yeast show that, for example, for cell cycle kinase cdc1 there are hundreds of substrates, namely, different proteins phosphorylated by this master switch during the cell cycle (126).

Whole pathways as a unit can become duplicated and diverted (29, 45, 99). For example, tryptophan and histidine biosyntheses (33, 49) in many organisms are catalyzed by homologous enzymes. In endosymbionts, specific enzyme families have duplicated and augmented. This includes ancient duplications supplying TIM barrel fold enzymes involved in metabolic supplies for the symbiotic host (e.g., triosephosphate isomerase itself and further isomerases of the carbohydrate metabolism) as well as kinase activities (ATP-binding folds). Examples of more recent duplications in endosymbionts are the ABC transporters (Bfl394 and Bfl396, a *iolE*-like and a *ycfU*-like gene) and the putative inner membrane proteins Bfl036 and Bfl037 (both belonging to the orthologous gene cluster COG0795).

In most organisms, "recruiting" enzymes from existing path-

ways is the most often observed behavior (17, 99, 107, 124), resulting in a mosaic or patchwork of homologous enzymes catalyzing reactions in distinct pathways. Such versatility has also been found for many *E. coli* small-molecule metabolism enzymes (122), e.g., in glycolysis and amino acid metabolism. Recruitment also seems to have played a major role in endosymbionts; at least, the most commonly observed enzyme folds such as the TIM barrel enzyme superfamily (17, 83) occur in different pathways, suggesting their widespread recruitment. Central pathways which were present in the last common ancestor and are found in all bacteria are also conserved in endosymbionts, e.g., translation, transcription, and core metabolism. Considering that also "*Candidatus* Blochmannia," *Buchnera*, and *Wigglesworthia* have been symbionts for tens of millions of years, this is a strong difference with respect to mitochondria and chloroplasts, where these central parts, except for the core of ribosome and translational apparatus with polymerases, have also been lost. Further additions include a couple of gram-negative-specific genes and enzymes.

The three endosymbiont genomes are clearly not yet minimal genomes, since for certain pathways further reduction can be conceived, specific protein duplications have occured, and they do not represent a minimal solution for other parts of the metabolism (e.g., amino acid metabolism). However, pathways are often so reduced that the bacteria depend for important parts on the host; e.g., there is respiratory metabolism but no citrate cycle and no ubichinone synthesis in *Buchnera*.

Good anabolic capabilities include mainly amino acids important for the host. Sulfur metabolism is retained at least in several endosymbionts. To a certain extent, however, there is genome amplification as a means of providing sufficient metabolic compounds for the symbiosis partner.

Endosymbiotic Bacteria: on the Way To Becoming Cell Organelles?

The endosymbiotic bacteria described in this review may at first sight show some similarities to organelles such as mitochondria and plastids, which also have extremely reduced genomes, reduction of the cell wall, replication control mechanisms different from those of normal eubacterial chromosomes, and a significant AT bias of their genomes (15, 123), possibly, the destiny of the insect endosymbionts may be to become some kind of new organelle (120). However, in contrast to organelles, which settle in virtually all cells of the host organism, the endosymbiotic bacteria described here are confined to very specialized habitats, the bacteriocytes, which are the result of a complex developmental pattern (13), and the ovaries. Therefore, although these associations are very old and at least "*Candidatus* Blochmannia" and some *Buchnera* species have direct access to the germ line, the endosymbiosis did not evolve to a generalized infection of all body cells. It is now recognized that many of the genes of the mitochondrial and plastid ancestors have moved to the nucleus of the host cell, and most proteins building up these organelles are derived from nucleus-encoded factors (in many cases of bacterial origin) (59, 123). However, more sequence data are required from the host animals to gain insights into the destiny of the many bacterial functions of the endosymbionts which are no longer found in their genomes and may have been transferred

to the host nucleus or simply were lost. In recent work, some evidence was found for a lateral gene transfer of the obligate intracellular parasitic bacterium *Wolbachia pipientis* to the genome of its host insect, a bean beetle (56). However, genome sequencing of a *Wolbachia* strain derived from *Drosophila melanogaster* did not reveal any lateral gene transfer between the host and the endosymbiont (1, 139), indicating that this is a very recent or spectacular event.

Concluding Remarks

The symbiotic associations described here are characterized by a mutual interdependence of both partners to allow the occupation of ecological niches with specific nutrition conditions for the host animals. This is accomplished by the provision of essential food supplements by the bacteria to their host animals. The animals, on the other hand, became indispensable for the bacteria by their adaptation to a stable environment inside the eukaryotic cell, which allowed the bacteria to substantially reduce their metabolic diversity and structural integrity. All three endosymbionts live in a similar habitat inside specialized eukaryotic cells, although differences in the evolution of the bacteria may not only be due to a different association time in the symbiosis but may also be due to differences in the specific habitat, i.e., cytosolic versus vacuolar localization of the bacteria. For example, the cytoplasm of eukaryotic cells may also contain antimicrobial agents, which may require appropriate defense mechanisms by the bacteria (42).

Figure 13 shows a comparison of the metabolic repertoire of the endosymbiotic bacteria with regard to building block biosynthesis as deduced from the bacterial genome sequences. In the future, efforts should be made to provide experimental data about the metabolic fluxes between the symbiotic partners. In addition to the characterization of the endosymbiont metabolism, the reduction in the number of structural compounds such as LPS should be investigated with regard to questions such as whether such potentially dangerous compounds were lost because of their toxicity or the lack of need for an intact cell wall in the protective environment inside the eukaryotic host. A fascinating question to be investigated in the future is the regulation of cellular functions in the endosymbionts which have retained only a very limited set of regulatory and signal transduction proteins (2, 35, 112, 119, 130). Moreover, mutual control mechanisms of the host and the bacteria are not known so far, although the intriguing fact of the missing DnaA replication initiation protein in the cytosolic bacteria "*Candidatus* Blochmannia" and *Wigglesworthia* may indicate a direct control of bacterial multiplication by the host organisms. Possibly, replication of *Buchnera*, which retained the DnaA protein despite its longer evolutionary interaction with the host, may be controled more indirectly by the animals because of its location within vesicles probably derived from the host. Finally, it is an open question whether the symbioses described here are stable in the long term of evolution, since further genetic leaching may be a serious threat to these symbioses. It is therefore interesting that in the obviously nutrition-based symbioses of *Buchnera* and *Wigglesworthia* secondary bacterial endosymbionts are frequently observed which in part might already provide a selective advantage for the infected animals or may do so after the degeneration of the
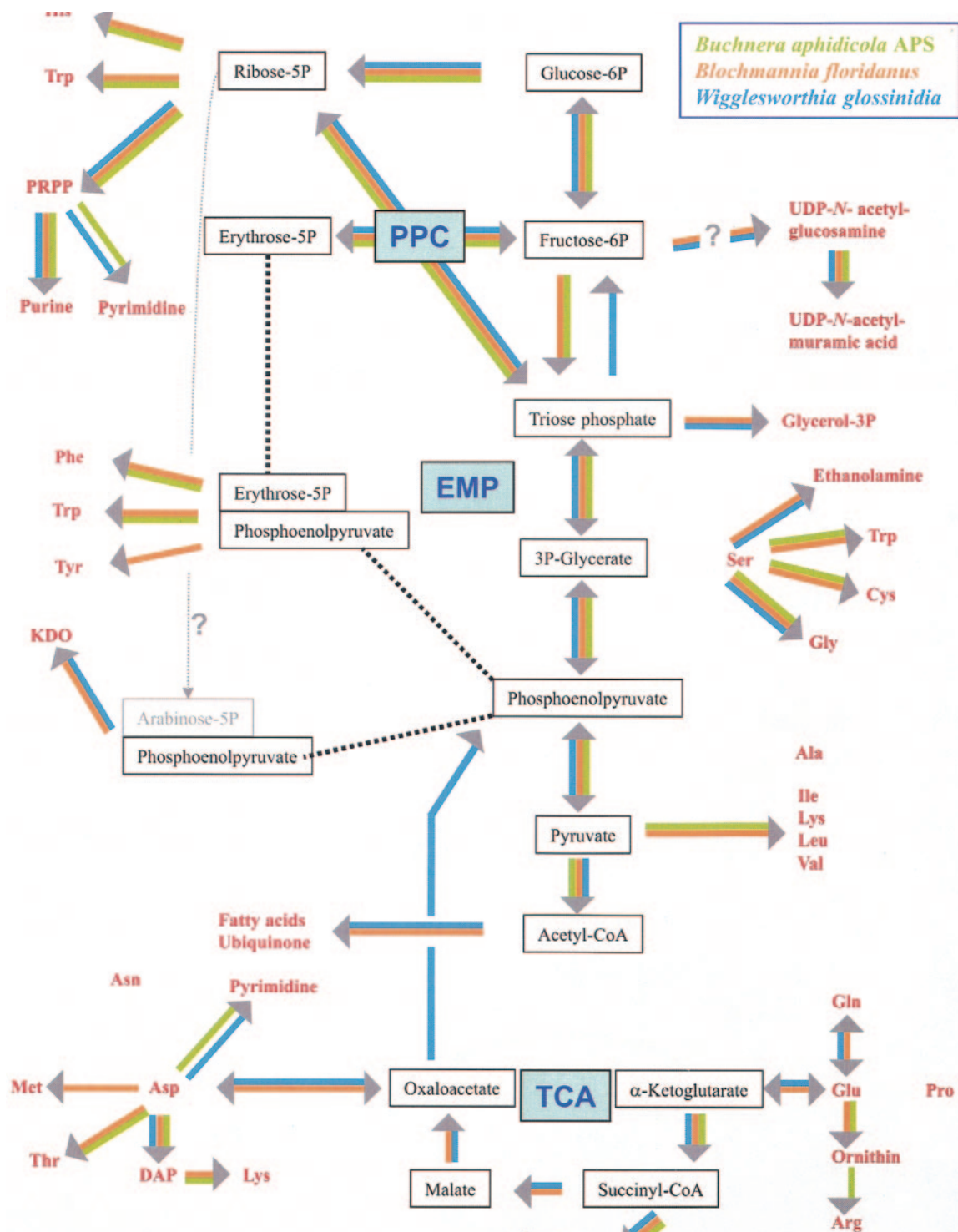
FIG. 13. Overview and comparison of the central intermediate metabolism of the endosymbiotic bacteria. Building-block biosynthesis of the endosymbiotic bacteria "*Candidatus* Blochmannia" (orange), *Buchnera* (green), and *Wigglesworthia* (blue) is shown as deduced from their genomic sequences. Important intermediates or end products are shown in red. Gray arrows indicate lack of the respective pathways. EMP, Embden-Meyerhoff-Parnass pathway (glycolysis); PPC, pentose phosphate cycle.

primary endosymbiosis (55). It is striking that in the "*Candidatus* Blochmannia"-*Camponotus* symbiosis, secondary endosymbionts are detected only rarely (105). This might be because the nutritional aspect of the symbiosis does not seem to be essential for the adult animals, which may have expanded the composition of their diet after the acquisition of the endosymbionts, which may have been relevant in early stages of the symbiosis.

The further characterization of such bacterium-host interactions will provide new insights into the physiological and molecular mechanisms involved in the stable integration of bacteria within a eukaryotic cell and will contribute to our understanding of molecular differences between symbiotic and pathogenic bacteria (36). Moreover, first applications based on such symbiotic interactions are emerging, e.g., with respect to pest control, as exemplified by new possibilities of vector control such as tsetse flies via the symbiotic bacteria (98).

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